CD28 and associated signalling elements of T lymphocyte signalling

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CD28 AND ASSOCIATED SIGNALLING ELEMENTS OF T LYMPHOCYTE SIGNALLING

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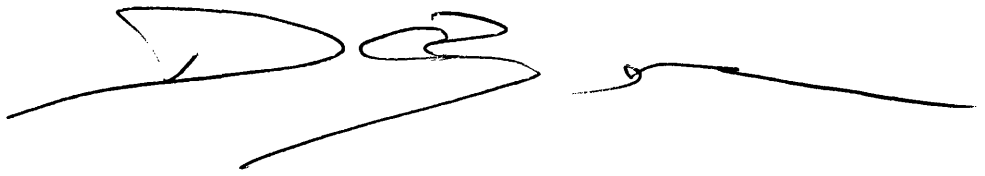

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This thesis is dedicated to
Darja

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Special thanks go to my wife Darja who softened the vicissitudes of science for me.

I would also like to thank my father who gave me the temperament to carry on and my mother who gave me the her support.

I look forward to the future when following the characterization of the myriad interactions between one protein and another, there will be fewer inexplicable mysteries regarding human motivation. Amazingly after 80000 years of existence we live only to chase the economic carrot and are encouraged only in rudimentary ambition.

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ABBREVIATIONS

-	negative
+	positive
ag	antigen
AICD	antigen induced cell death
A/N SMase	acidic/ neutral sphingomyelinase
AP1	activation protein-1
APC	antigen presenting cell
ATP	adenosine triphosphate
$Ca^{2+}i$	calcium ions
CAPK	ceramide activated protein kinase
CAPP	ceramide activated protein phosphatase
CC	CD28 transfected CHO
CDP	cytidyl diphosphate
CHO	Chinese hamster ovary cell
CP	phosphocholine
CPM	counts per minute
CsA	cyclosporin A
CTLA4	cytotoxic T lymphocyte-associated antigen-4
DAG	diacylglycerol
DHS	dihydrosphingenine
DMEM	Dulbecco's modified Eagles medium
DMS	dimethylsphingenine
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ERK	extracellular-signal regulated kinase
FACS	fluorescent activated cell sorter
FADD	Fas associated death domain-protein
FCC	Fyn transfected CD28 transfected CHO
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL	fluorescence
FMNC	phenylalanine methionine asparagine cysteine (mutant SH2 binding motif of CD28)
FSC	forward scatter
GAB	glutathione agarose bead
GAP	GTPase activating protein
GDP	guanine diphosphate

gpt	guanine phosphoribosyl transferase
GRB2	growth-factor receptor bound-protein 2
GST	glutathione S-transferase
HBS	Hepes buffered saline
HLA	human leukocyte antigen
ICAM	intracellular adhesion motif
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IP3	inositol 1,4,5 -triphosphate
IRS	insulin receptor substrate
IVK	in vitro kinase
JNK	c-Jun amino terminal kinase
kDa	kilo Dalton
LCC	Lck transfected CD28 transfected CHO
LFA-3	Leukocyte Function-associated Antigen-3
lysoPC	monoacylated phosphatidylcholine
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
myr	myristyl
NFAT(c)	nuclear factor of activated T cell (cytosolic-component)
NFkB	nuclear factor kappa beta
NGF	nerve growth factor
pYMNM	phosphorylated tyrosine methionine asparagine methionine, the CD28 SH2 binding motif for interaction with PI3K
PA	phosphatidic acid
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PI	propidium iodide
PI3K	phosphatidyl inositol 3-kinase
PI_nP	phosphatidyl $inositol_n$ phosphate
PKA/ B/ C	protein kinase A/ B/ C
PLC/ D	phospholipase C/ D
PMA	phorbol 12-myristate 13-acetate
PTK	protein tyrosine kinase

PTPase	protein tyrosine phosphatase
Rb(P)	retinoblastoma-protein (phosphate)
RIP	receptor-interacting protein
S6K	$p70^{S6\ kinase}$
SAPK	stress activated protein kinase
SH2/3	src homology domain 2/ 3
SHC	src homology and collagen adapter protein
SOS	son of sevenless
SPP	sphingosine-1-phosphate
SSC	side scatter
TBS(N)	tris buffered saline (with NP40)
TCR	T cell receptor-complex
Th	T helper
TNF	tumour necrosis factor
TOR	target of rapamycin
TRADD	TNF receptor 1 associated death domain-protein
TRAF2	TNFR-associated protein 2
VDJ	variable diversity joining
ZAP70	zeta associated protein
ZCC	ZAP transfected CD28 transfected CHO

Abstract

Signals generated following stimulation of CD28 and the TCR synergize resulting in T cell proliferation, cytokine secretion and cell survival. Investigations of the mechanism by which CD28 costimulates the activation of a resting T cell have not identified an obligate effector responsible for all of the effects occurring subsequent to the ligation of CD28. The role of proximal signalling molecules in CD28 signalling was examined. In T cell models it was observed that lck and fyn but not ZAP70, in response to ligation of CD28 by CD80, increased the phosphorylation of CD28 and PI 3-kinase. Although fyn and lck are tyrosine kinases, changes in tyrosine phosphorylation levels were small compared to increases in the phosphorylation of tyrosine/ serine / threonine residues on CD28 and PI 3-kinase. This would suggest that fyn and lck facilitate the recruitment of additional molecules to CD28 and ligation of CD28 by CD80 lead to activation of an acidic sphingomyelinase activity. However neither sphingomyelinase, nor the products of sphingomyelin hydrolysis by sphingomyelinase, C2 ceramide and phosphocholine replaced CD80 as a costimulus for resting T cell proliferation . Indeed sphingomyelinase/ C2 ceramide inhibited costimulated T cell proliferation. The inhibition of proliferation caused by sphingomyelinase/ C2 ceramide was accompanied by a delay in the upregulation of surface expression of the activation markers CD69 and CD25. This delay was dependent on the primary proliferative stimulus being αCD3 mAb rather than the PKC agonist, PMA. In contrast PMA, CD80-costimulated T cell proliferation was inhibited by C2 ceramide and this would suggest that C2 ceramide has more than one mechanism of altering T cell responses. Analysis of a distal substrate of CD28, JNK, revealed that CD3, CD28 costimulation of resting T cells did not phosphorylate c-Jun, a substrate of the JNK family members, as markedly as PMA. Despite the ability of sphingomyelinase/ C2 ceramide to inhibit T cell proliferation, JNK activation was unaltered. It may be suggested that the inhibition of proliferation due to sphingomyelinase/ C2 ceramide in costimulated T cell cultures was not effected by inhibition of c-Jun phosphorylation, an event necessary for the formation of AP1 , a transcription factor necessary for activation of the IL2 promoter.

Chapter 1

Introduction

1 The Immune System

The immune system comprises an innate and an adaptive component. The innate immune system defends the body by the use of skin, epithelial linings e.g. mucous and cilia, sneezing and swallowing which serve to keep matter not generated by host DNA, i.e. foreign matter, external from the body. Should the innate immune system be overcome or bypassed and foreign matter enters the body after crossing cellular membranes, an adaptive immune response may be generated. In this case the foreign matter is known as an antigen. Antigenic matter may also be generated within the body and differentiation between self and non-self antigens is an important part of the immune system (Clarke, 1980).

1.01 Antigen Presentation

The adaptive immune system comprises a number of elements, some give it "memory" i.e. the ability to mount a quick reaction against recurrent infections, while other parts are involved in recognition of new antigens and clearance of these from the body. Thus a sophisticated array of cell types is necessary to implement the various functions of the immune system. In the humoral arm of the adaptive immune system, antigen circulating in the blood or lymphatic system is bound by antibodies (immunoglobulins) secreted by B cells. This is an antigen dependent step and ultimately leads to clearance of the bound antigen-antibody complex from the body. In a tissue based or cellular response, antigens are processed by a number of specialised cells known as antigen presenting cells (APCs) including B cells, dendritic cells and Langerhan cells (June et al., 1994; Damle et al., 1992). These cells are positive for the human leukocyte antigen (HLA-DR, DQ, DP) alleles and serve to immobilise the antigen on the cell surface in a complex with a major histocompatibility (MHC) molecule. Antigenic proteins, phagocytosed by macrophage-like cells, are degraded into smaller peptides. These are incorporated into the peptide binding grooves of MHC molecules and presented on the cell surface of the APC. The MHC-antigenic signal in combination with accessory signals from APCs provide the stimuli which T cells require to mount a functional immune response.

1.02 The Role of T cells

T cells may be classified according to the expression of CD4 or CD8 glycoproteins on their cell surface membranes. Usually CD8 cytotoxic T lymphocytes (CTLs) recognise cytosolic or nuclear-derived antigen associated with MHC class I and eliminate virally infected cells or tumour cells. CD4 T helper (Th) cells respond to antigen associated with MHC class II and generate cytokine signals which stimulate a range of other immune responses (Germain and Marguiles, 1993). CD8 and CD4 bind invariant regions of their respective MHC molecules.

The delivery of antigen-APC signals to T cells induces the activation of the T cell, resulting in cytokine gene upregulation and secretion from the T cell. Human $CD4^+$ T cells can be divided into four functional subsets Thp, Th0, Th1 and Th2 according to their pattern of cytokine secretion. T helper precursors (Thp) are mainly limited to the production of interleukin (IL) 2. Activated Thp cells most likely differentiate initially into Th0-like cells producing IL2, IL4 and granulocyte/ macrophage-colony stimulating factor (GM-CSF), but not tumour necrosis factor (TNF)β or IL5. Subsequent maturation can lead to either, Th1 like cells secreting IL2, interferon (IFN)γ, TNFβ and GM-CSF or Th2 like cells which produce IL4, IL5, IL10 and low levels of GM-CSF. Functionally the Th1 phenotype is associated with cell-mediated immunity and Th2 with humoral responses (Semnani et al., 1994). IL2 is responsible for autocrine proliferation of the T cell involved in the antigen dependent activation event as well as induction of paracrine lymphocyte proliferation. Within a T cell there are regulatory elements to prevent the activation of the cell due to inappropriate antigen presentation, such as a requirement for a second costimulatory signal in addition to stimulation of the TCR. However sometimes these processes are ineffectual in discriminating between self and non-self antigens and autoimmune disorders arise. An understanding of the processes driving T cell activation and the biochemical pathways involved is essential if intervention therapies are to be applied in treatment of virulent pathogens or suppression of allograft rejection, both of which involve T cells (Clarke, 1980).

An alternative classification of circulating T cells may also be made based upon their requirements for signals in order to mount an effective immune response. Resting T cells, characterised by being non-proliferative unless stimulated by a combination of antigenic-derived and accessory, or costimulatory, signals may be subdivided into naive and memory resting T cells. The naive population bear the surface phenotype $CD45RA^+$ CD45RO- and the memory population the converse. It seems likely that both of these subpopulations arise from naive T cells and after encounter with antigen become $CD45RO^+$ (Semnani et al., 1994). Thus naive T cells are classed as those which have not encountered antigen previously, while memory T cells have previously bound antigen but are no longer in an activated state. Both require stimulation additional to antigen to proliferate or become activated. A third type of T cell also exists which has encountered antigen and is deemed an antigen-activated T cell which requires minimal stimulation to cause proliferation (Damle et al., 1992).

1.1 T cell Activation

An *in vivo* T cell immune response occurs following an antigen-induced clonotypic expansion of T cells in conjunction with accessory signals from APCs. The expansion of the antigen-responsive T cell clone is facilitated by the upregulation of IL2 gene expression in the clone and secretion resulting in clonal expansion. When coupled with expression of a range of cytokines from the activated T cell, other immune cells migrate to the area involved, become activated as well and perform their respective functions. T cell proliferation and activation frequently occur together. Activation, which may be regarded as an upregulation of cytokine gene expression (Granelli-Piperno and Nolan, 1991; Thompson et al., 1989), is concurrent with increased expression of the IL2 receptor, CD25, and the early T cell activation marker CD69 (Semnani et al., 1994). However proliferation and activation are not indistinguishable responses in T cells. Proliferation to CD3 signals alone in activated T cells was not augmented by accessory signals and yet accessory signals did cause a 2 fold increase in RNA metabolism. In fact cytokine expression and secretion was upregulated over 50 fold with no marked effect on proliferation caused by the accessory signal (Thompson et al., 1989). Conversely proliferation of CD3 stimulated memory T cells was induced by some accessory signals without concomitant IL2 upregulation i.e. without activation of the T cell (Damle et al., 1992).

1.11 Structure and Function of the T cell Receptor Complex

T cell activation is partially controlled in an antigen-dependent manner and a clonotypic complex of molecules expressed on the surface of a T cell facilitates recognition of antigen and passage of signals to the T cell nucleus, whereupon a range of genes is upregulated resulting in an immune response. The T cell molecules involved in receiving and transducing the MHC-antigenic signal are collectively known as the T cell receptor complex (TCR). The TCR, a glycoprotein, comprises an antigen recognition heterodimer which on 95% of peripheral T cells is composed of $\alpha\beta$ chains. The $\alpha\beta$ chains possess variable (V), joining (J) and constant (C) regions with the β chain containing a diversity domain (D) (Davis and Bjorkman, 1988). The V$J\alpha$ and VDJβ domains contain three

hypervariable regions, one of which is proposed to interact with the antigen and the other two with the MHC molecule (Davis and Bjorkman, 1988). Gene rearrangement and recombination events of the several VDJ alleles generates a large TCR repertoire.

Autoreactive T cells recognise self antigen in the context of self MHC molecule and may be pathogenic. However clonal deletion of developing autoreactive T cells occurs in the thymus by a process which is poorly understood, although believed to be the primary mode of deleting autoreactive T cells before release of thymocytes to the peripheral circulatory system (Davis and Bjorkman, 1988). Following TCR αβ ligation by antigen-MHC molecules an associated complex of CD3 and the ζ family of dimers are responsible for transducing the APC derived stimulus into the T cell. CD3 comprises γ, δ and ε chains while the ζ dimers are ζζ, ζη or ζγ (Irving and Weiss, 1991) (fig. 1.1).

1.12 TCR Proximal Signalling Events

An early event after TCR ligation either by antigen or antibodies against the TCR, is the tyrosine phosphorylation of certain proteins in the T cell. This response is rapid (within 30 seconds) and short in duration, although alone does not activate the T cell *per se* (August et al., 1994; Harlan et al., 1995; Lu et al., 1994; Mustelin, 1994; Robey and Allison, 1995). Unlike many other receptors involved in cellular proliferation, the TCR has no intrinsic kinase activity in any of its subunits. Thus it must recruit various kinases to its cytoplasmic domains. Tyrosine motifs serve as substrates for cytosolic protein tyrosine kinases (PTKs) such as those of the *src* and *syk* families. Members of these kinase families reported to associate with the TCR include $p59^{fyn}$ and $p56^{lck}$ for the *src* PTKs and syk and $p70^{ZAP}$ kinase (ZAP70) for the *syk* PTKs (Mustelin, 1994). The involvement of these kinases revolves around a conserved amino acid sequence, the intracellular T cell activation motif (ITAM), $YXX(L/I)X_{7-8}YXX(L/I)$ where X is any amino acid, found in three copies on ζ chains and one copy on each of the CD3 subunits. Two isoforms of fyn exist which are the products of mutually exclusive splicing of alternate seventh exons (Appleby et al., 1992). Transcripts utilizing exon 7B accumulate primarily with the T lymphocyte population and exon 7A transcripts are localized in other tissues, particularly the brain. Respectively these isoforms are referred to as $p59^{fynT}$ and

$p59^{fynB}$ (Appleby et al., 1992). In this study reference is only made to $p59^{fynT}$ (fyn). The involvement of fyn in T cell signalling appears to be stage specific. Interestingly while thymocytes from fyn null mice show reduced proliferation, splenocytes reaquire a proliferative response to stimulation of CD3 and PMA (Appleby et al., 1992). This would imply that the significance of fyn in T cell signalling is decreased as T cells mature. In patients with an autosomal recessive form of severe combined immunodeficiency disease, a double mutation in the kinase domain of ZAP70 was discovered. These mutations were absent from normal siblings and disease free parents. The effect of mutated ZAP70 kinase on T cell function following TCR ligation was a decrease in cellular tyrosine phosphorylation and calcium mobilization (Chan et al., 1994). Therefore it may be suggested that ZAP70 contributes to TCR signalling and T cell function.

The order of PTK recruitment to the TCR is not defined and may indeed vary depending upon the type of stimulation and intracellular environment. While syk associates constitutively with CD3 i.e. in an activation independent fashion, lck and fyn are brought into association following ligation of the TCR. Fyn associates with CD3 while lck associates with CD4 (a transmembrane protein which stabilises TCR-MHC class II-antigen interactions). Both lck and fyn are believed to phosphorylate ITAMs. Following the dual tyrosine phosphorylation of the ITAM, ZAP70 associates with the TCR (Rudd et al., 1994; Hatada et al., 1995; Straus and Weiss, 1993). Subsequently it activates phospholipase Cγ1 (PLCγ1) which catalyzes the conversion of $PI_{4,5}P_2$ to inositol triphosphate (IP_3) and diacylglycerol (DAG). These products are second messengers involved in raising intracellular calcium levels and protein kinase C (PKC) activation (Vance and Vance, 1991). Both of these events ultimately result in induction of transcription factors such as the nuclear factor of activated T cells (NFAT), c-Fos and c-Jun which have a role in upregulating IL2 expression (Robey and Allison, 1995; Liu, 1993).

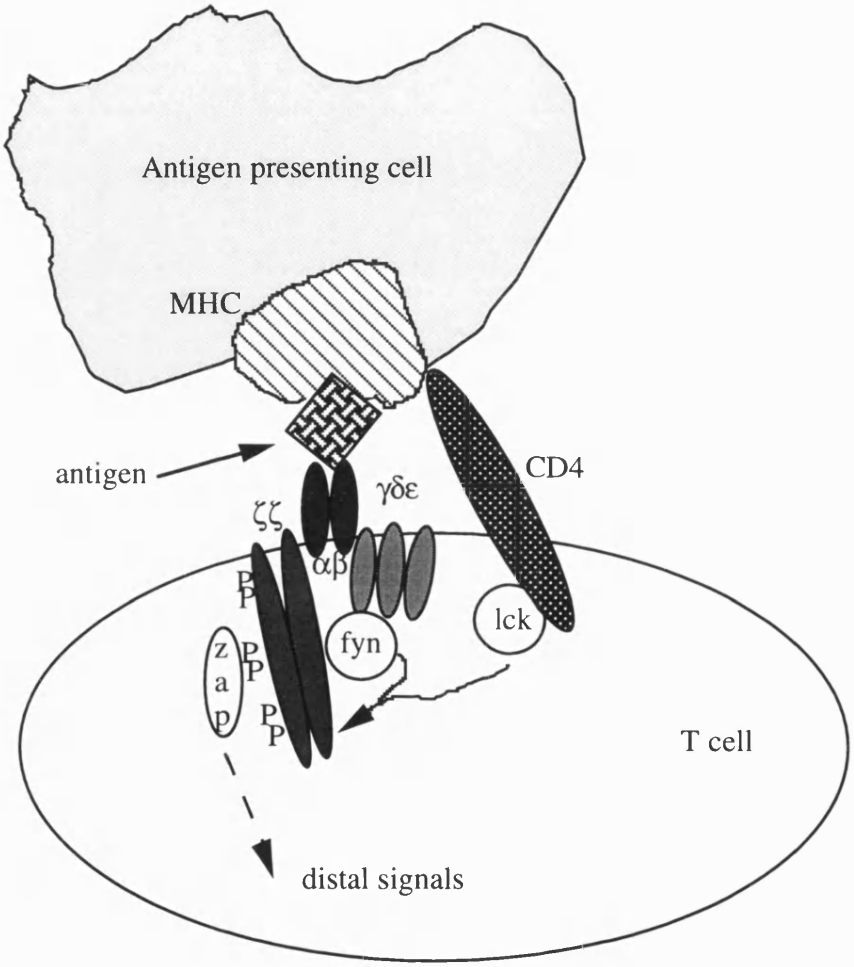


Fig. 1.1: Proximal Signals Associated with TCR Ligation

Encounter with an APC bearing antigen, which can be recognised by the TCR, elicits activation of a number of proximal signalling molecules facilitating T cell activation and proliferation.

1.13 Structure, Function and Regulation of Cytosolic PTKs

A *src* kinase has two sites which may be phosphorylated. The carboxyl terminal site is negatively regulated by another tyrosine kinase $p50^{csk}$ (csk) (Nada et al., 1993; McFarland et al., 1992) and when these kinases are associated with the TCR, positive regulation arises from the action of a transmembrane protein tyrosine phosphatase, CD45. While src PTKs are inhibited by csk, CD45 becomes activated by a csk-induced phosphorylation of two tyrosine residues (Nada et al., 1993). Dephosphorylation of the regulatory C terminal tyrosine residue (Autero et al., 1994) of a src family PTK by CD45 is proposed to release an intramolecular binding of the upstream SH2 domain of the src family PTK, leaving the kinase motif available for activation (Mustelin, 1994) (fig. 1.2). However what phosphorylates and activates this kinase motif is unknown, even in the much studied TCR signalling cascade.

The mechanism(s) by which PTKs are recruited to the TCR is unclear, although in src family PTKs a unique amino acid (N)-terminal moiety, myristate (myr)-Gly-X-X-X-Ser/Thr/Cys is believed to play a role in association of the PTK with substrates and determining specificity of the PTK (Resh, 1994). During translation of a src PTK, an amino terminal initiator methionine residue is removed by methionine aminopeptidase and glycine becomes the first amino acid in the sequence. A covalent bond between the N terminal glycine and a myristate fatty acid catalyzed by N-myristyl transferase is stable for the half-life of the polypeptide i.e. is essentially irreversible (Resh, 1994). The myristyl moiety present in src kinases, is absent in ZAP70, and dependent on the absence of other suitable hydrophobic sites, may prevent ZAP70 from membrane localization. As ZAP70 is involved in TCR signalling (Straus and Weiss, 1993), it is evident that a myristate residue is not an obligate requirement for all proximal signalling molecules.

The N terminal sequences of src family PTKs may supply specificity in kinase interactions with substrates and how they dock with other proteins.

	3 6 9 12 15
Fyn has the sequence:	myr G **C** V Q **C** **K** D **K** E A T **K** L T F
and lck has the sequence:	myr G **C** V **C** S S N P E D D W M E N

The plasma membrane inner leaflet comprises some 30% negatively charged lipids, head groups extending to the hydrophilic cytosol, which are mainly phosphatidylserine and phosphatidylinositols. Thus a possibility exists that polybasic residues on molecules associating with the plasma membrane may stabilize their localization. For example fyn has three lysine (K) residues (indicated in bold type) which may fulfil this role and likely would have an electrostatic interaction with negatively charged head groups of membrane lipids. Lck lacks these and so other mechanisms to stabilize anchorage of lck may operate. For example dual acylation of cysteine residues (indicated in bold type) with the 16 carbon fatty acid palmitate has been demonstrated (Shenoy-Scaria et al., 1993; Paige et al., 1993) on lck and fyn while many α subunits of G proteins which are also recruited to the membrane share the sequence myrGC with 7 of 9 src family PTKs which have this sequence (Resh, 1994). Palmityl thioesterases can cleave the thioester bond between cysteine and palmitate (Camp and Hofmann, 1993) providing a mechanism by which the stability of anchorage of lck or fyn may be undermined allowing the PTKs to interact with multiple membrane bound targets.

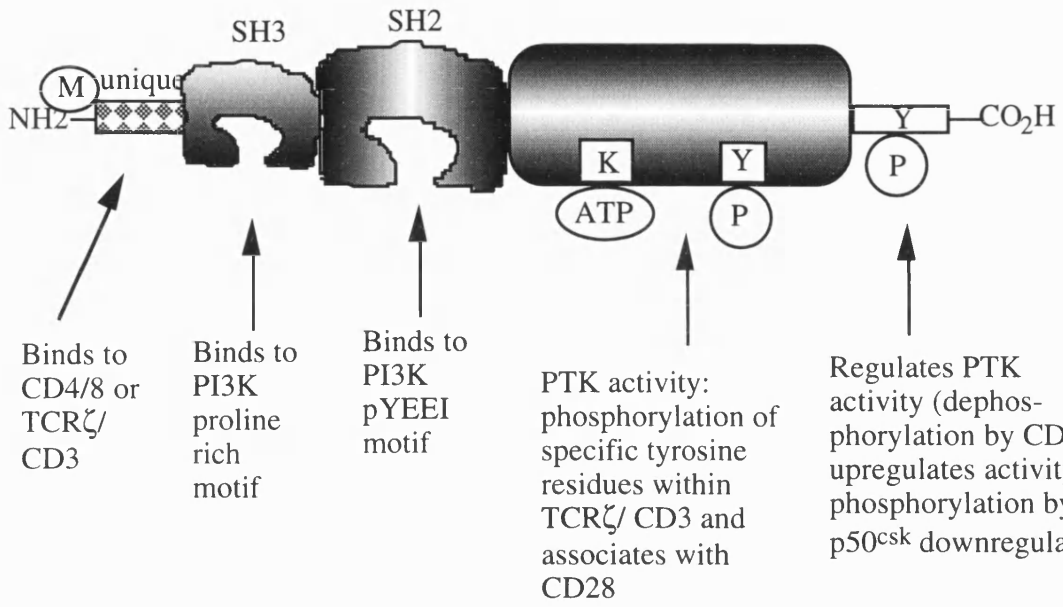


Fig. 1.2: Structure of the *src* family Protein Tyrosine Kinases

Structure of *src* family PTKs, $p56^{lck}$ and $p59^{fyn}$. The N-terminus (70-80) residues contains largely unique residues except for the myristate (M) moiety. Lck interacts with CD4 and CD8 within 10-30 residues of the N terminus. The interaction of fyn with CD3 and ζ is mediated by the first 10 residues. The N terminus is followed by an SH3 and SH2 domain with moderate homology between src family PTKs. The highly conserved kinase domain possesses an autophosphorylation site at tyrosine (Y) 394 for lck and 420 for fyn, and an ATP binding lysine, K residue. The C terminus possesses a negative regulatory site (Y505 for lck and Y531 for fyn) (Rudd et al., 1994).

1.2 Costimulatory Effects of CD2, CD28 and LFA-1

A number of other molecules which play a role in T cell activation have been identified. The receptors CD2, CD28 and LFA-1 (CD11a/ CD18) which are expressed on T cells may bind, respectively, the ligands LFA-3 (CD58), B7s (CD80 or CD86) and ICAM-1 (CD54). LFA-3, B7s and ICAM-1 are expressed on APCs (Parra et al., 1993). With at least three pairs of ligand-receptor interactions sufficient to costimulate T cells, a possibility of functional redundancy exists. However each interaction has distinct effects on T cell activation and proliferation. Studies comparing the effects of ICAM-1, LFA-3 or CD80 (and αCD28 mAb 9.3) revealed differential responses from αCD3 mAb stimulated T cells in the presence of these ligands (Semnani et al., 1994; Damle et al., 1992; Sansom et al., 1993).

1.21 ICAM-1

ICAM-1, which may ligate the T cell receptor LFA-1, costimulated naive T cell proliferation and IL2 production (Semnani et al., 1994). Repeated costimulation of separate populations of either naive or memory T cells lead to the production of GM-CSF and IFNγ and greater levels of these cytokines were recorded after the primary stimulation (Semnani et al., 1994).

1.22 LFA-3

LFA-3, the ligand for CD2, also costimulated memory T cell proliferation, secretion of IL2 and IFNγ and upon further stimulation IL5 production was induced. However LFA-3 could not costimulate naive T cell proliferation, and its mode of action appeared to be dependent on a close association with the TCR (Semnani et al., 1994; Sansom et al., 1993).

1.23 CD80 and CD86

CD80 and CD86 are ligands of the B7 family, expressed on APCs, which bind CD28. The B7s and CD28 are members of the larger immunoglobulin superfamily. CD80 (B7 or B7-1) is a 44-54 kDa glycoprotein while CD86 (B70 or B7-2) (Allison, 1994) is a 70

kDa glycoprotein, expressed on activated B cells, dendritic cells and monocytes (Harlan et al., 1995; Umlauf et al., 1993), although CD86 is also detectable on freshly isolated monocytes (Azuma et al., 1993). Furthermore CD86 surface expression is rapidly elevated after activation of APCs and may perhaps be the primary ligand for CD28. In addition a 3 to 4 fold greater affinity between CD80 and CTLA4 over that of CD86 and CTLA4 has been demonstrated (Greene et al., 1996). Due to the correlation in the pattern of expression between CD86 and CD28 compared to CD80 and CTLA4 surface expression, it might be expected that there is a mutually exclusive functional relationship between these pairs of ligands and receptors. However in the experiments of Kuchroo et al (Kuchroo et al., 1995) it was noted that costimulation of murine T cells by CD80 or CD86 lead to the induction of a Th1 or a Th2 cytokine profile respectively, therefore both ligands are capable of activating T cell responses from activated T cells. In this study the ligands are referred to as either CD80 or CD86, unless there was ambiguity in a report as to which member of the B7 family was involved whereby the generic term B7 was employed. In addition in some figures e.g. figs 4.4b and 4.5 the term B7 refers to CD80.

1.24 CD28 and CTLA4

CD28 is a member of the immunoglobulin superfamily expressed on 95 % of CD4 T cells and 50% of CD8 T cells (Lu et al., 1992). As a monomer it has a predicted molecular weight of 23 kDa but after glycosylation migrates under reducing conditions at 44 kDa. It exists as a homodimer and is a transmembrane receptor for CD80 and CD86 (Aruffo and Seed, 1987). CD28 binds both ligands with comparable affinity (Greene et al., 1996) although CD86 shows upregulated surface expression before CD80 and therefore CD86 would initially interact with CD28 (June et al., 1994).

A homologue of CD28, the cytotoxic T lymphocyte antigen-4 (CTLA4) has upregulated expression on activated T cells (Walunas et al., 1994) in parallel to the expression of CD80 on activated APCs (June et al., 1994). In mice lacking CTLA4, fatal lymphoproliferative disorders arise rapidly including multiorgan lymphocytic proliferation and tissue destruction by 3-4 weeks of age (Tivol et al., 1995). Therefore

CTLA4 appears to have a regulatory role in T cell biology. Accordingly a CTLA4Ig fusion protein has been used to prevent B7-CD28 interaction, inducing the prolonged acceptance of murine skin and cardiac allografts (Lakkis et al., 1997; Larsen et al., 1996). CTLA4 is generally believed to limit the effect of CD28 in costimulation, either through competition for B7 ligands or induction of anti-proliferative pathways (Harlan et al., 1995; Robey and Allison, 1995; Stein et al., 1994; Schneider et al., 1995b; Walunas et al., 1994). For example CTLA4 is capable of delivering negative regulatory signals to costimulated T cells resulting in reduced or delayed cell cycling and lowered CD25 (IL2 receptor) and CD69 expression (Krummel and Allison, 1996) as well as decreased IL2 secretion from T cells (Krummel and Allison, 1996; Walunas et al., 1996). CTLA4 has the ability to bind CD80 with twenty fold higher affinity than CD28 (Linsley et al., 1991) but unlike CD28, which is expressed on resting and activated T cells, CTLA4 surface expression is restricted to activated T cells (Walunas et al., 1994) and is mainly localized intracellularly (Leung et al., 1995). On the cytoplasmic domain of CTLA4 an intracellular localization motif was initially identified as T^{161}TGVYVKMPPT (Leung et al., 1995) and later Y^{165} was found to be responsible for associating with the μ2 subunit of the plasma membrane-associated adapter complex AP2 causing internalisation of CTLA4 (Shiratori et al., 1997). Interestingly CTLA4 only associated with AP2 when Y^{165} was unphosphorylated. By contrast the PTPase Syp associates with CTLA4 when Y^{165} is phosphorylated (Marengere et al., 1996) and Syp also demonstrated PTPase activity against the src homology and collagen adapter protein, $p52^{SHC}$ (Marengere et al., 1996). These studies support the possibility that CTLA4 may negatively regulate T cell activation by dissociating SHC from complexes of GRB2 and Sos, thereby preventing Ras activation following TCR ligation (Marengere et al., 1996). Therefore CTLA4 function is controlled by the tyrosine phosphorylation of Y^{165} whereby in its unphopshorylated form, CTLA4 associates with AP2, is internalized and is therefore incapable of interacting with its ligands CD80 and CD86. By contrast when phosphorylated, Y^{165} associates with Syp, a PTPase which may inhibit TCR-derived Ras and MAPK pathway activation. A question remaining to be answered is what is the identity of the kinase(s) which phosphorylate Y^{165} , a residue which is critical to CTLA4 function. By contrast CD28 is mainly expressed on the T cell surface although it

undergoes a transient downregulation following ligation at an mRNA level by 4 hours and surface expression from 12 to 24 hours (Linsley et al., 1993).

1.25 Functional Effects of CD28 Stimulation

CD28 although expressed on T cells appears to contribute to B cell activation as well. For example $CD28^{-/-}$ mice had depressed levels of serum immunoglobulin in addition to the reduced proliferation of, and IL2 production from, splenocytes (Shahanian et al., 1993). Therefore while CD28 costimulates T cell activation, it may also stimulate B cell activation. This may be mediated by the interaction of CD28 with CD86 which has a relatively longer cytoplasmic domain compared to CD80, presenting a greater opportunity for expression of localization motifs. Indeed three PKC phosphorylation sites are expressed on the cytoplasmic domain of CD86 (June et al., 1994) which possibly contribute to activation of immunoglobulin gene promoters in B cells. The role of CD28 in regulating T cell activation revealed that antibody ligation of CD28 in combination with TCR or TCR-like signals resulted in the production of a range of cytokines including IL2, IFNγ, TNFα, lymphotoxin (LT) and GM-CSF from T cells (Thompson et al., 1989) and therefore CD28 may modulate cell cycle progression through IL2, lysis of tumour cells through TNFα and LT, anti-viral and anti-tumour responses of T cells through IFNγ and multilineage haematopoiesis through GM-CSF (Thompson et al., 1989). The experiment also demonstrated a dichotomy between proliferation and activation of T cells. While resting T cells require costimulation in order to proliferate and become activated i.e. upregulate cytokine expression, the cells in the above study were likely to be a mixture of resting and antigen-primed T cells, due to T cell proliferation and cytokine secretion to αCD3 signals alone. A separation of proliferation from cellular activation was apparent in that although CD28 did not increase levels of ^{3}H thymidine incorporation into DNA (which increases as cells proliferate), it doubled the levels of labelled uridine incorporated into RNA in the T cell culture. Therefore CD28 stimulated gene expression, not DNA replication, in activated T cells. The study mentions that the effect of CD28 on proliferation *per se* may only be seen with suboptimal stimulation of CD3. However the effect of CD28 on gene activation was not dependent on suboptimal CD3 signals. Therefore CD28 showed capability to augment T

cell responses in resting and activated T cells as it costimulated proliferation and activation of resting T cells (Damle et al., 1992) and augmented RNA synthesis in activated T cells (Thompson et al., 1989).

An effective immune response *in vivo* requires more than a single type of costimulatory interaction. The binding of ICAM-1 to LFA-1 appears to have the capability of causing Thp cells to become activated and differentiate to firstly Th0 cells and later Th1 and to cause activation of naive T cells which neither CD28 nor CD2 derived costimulations were capable of in the studies discussed above (Semnani et al., 1994; Damle et al., 1992). However CD28 and CD2 were able to transduce activation signals to resting memory cells and it has been proposed that the regulatory and effector functions of T cells are mainly carried out by memory T cells (Sanders et al., 1988) in which case CD28 and/ or CD2 may be more significant players in T cell responses. Accordingly signals transduced through CD28, more than other costimulatory receptors, resulted in very high IL2 production, IL2 receptor expression and proliferation of memory T cells (Damle et al., 1992) illustrating the key role of CD28 in coordinating T cell responses in an immune response. An apparent similarity in responses of CD28 and CD2 after interaction with their respective ligands may be explained by the differences in subsequent cytokine profiles to which each contributes. In the study of Thompson which ligated CD28 through the use of αCD28 mAb, a Th1 range of cytokines (IL2, IFNγ, TNFα, LT and GM-CSF) were produced (Thompson et al., 1989), whereas for CD2 the converse was found. That is CD2 transduced signals leading to production of Th2-type cytokines e.g. IL5, and low levels of GM-CSF (Semnani et al., 1994; Damle et al., 1992). However a separate study indicated that secretion of the Th1 cytokine IFNγ was independent from CD28 ligation and that a Th2 cytokine response was depressed in the absence of CD28 -/- mice (Rulifson et al., 1997). It would appear from these data and that of Kuchroo (Kuchroo et al., 1995), i.e. that CD80 and CD86 induce respectively Th1/ Th2 responses, that CD28 is capable of stimulating both responses.

CD28 also has other functions which control the fate of the cell upon which it is expressed. Ligation of the TCR on a resting T cell by antigen or antibody in the absence

of accessory signals normally supplied by an APC does not cause cellular activation or proliferation. In fact the cell becomes hyporesponsive i.e. anergized to subsequent combinations of antigenic and accessory signals (Schwartz, 1993). While CD28 may prevent anergy, stimulation of CD2 by LFA-3 cannot (Sansom et al., 1993). The effects of stimulating the TCR/ CD3 and/ or CD28 are summarized in figure 1.3 and the prevention of anergy may be facilitated by the differential association of fyn, lck and ZAP70 with the TCR (Boussiotis et al., 1996). Antigenic stimulation of $CD4^{+}$ve T cell clones resulted in their anergy, tyrosine phosphorylation of TCRζ and association of TCRζ with fyn. By contrast costimulation of these cells resulted in the tyrosine phosphorylation of TCRζ and CD3ε and their association with lck and later ZAP70, but not fyn (Boussiotis et al., 1996). Therefore it may be possible that B7 stimulation of CD28 leads to signals preventing anergy by inducing differential association of PTKs with the TCR, although ligation by CD80 or CD86 induced comparable tyrosine phosphorylation profiles (Boussiotis et al., 1996).

T cell survival is also regulated by CD28. T cells are uniquely capable of undergoing a form of programmed cell death (apoptosis) known as activation induced cell death (AICD) which occurs as a result of repeated antigenic stimulation (Van Parijs et al., 1996). This occurs following coexpression of Fas (CD95) and the Fas ligand (FasL). FasL expression on T cells (Lynch et al., 1995; Anel et al., 1994) leads to AICD following stimulation of Fas which can be prevented by CD28-mediated signals (Van Parijs et al., 1996). The anti-apoptotic function of CD28 is likely due to its upregulation of survival factors such as bcl-X_L (Boise et al., 1995; Mueller et al., 1996) or possibly components of the transcription factor NFkB, c-Rel and Rel A, which were reported to antagonise TNF-induced apoptosis (Liu et al., 1996; Lin et al., 1997).

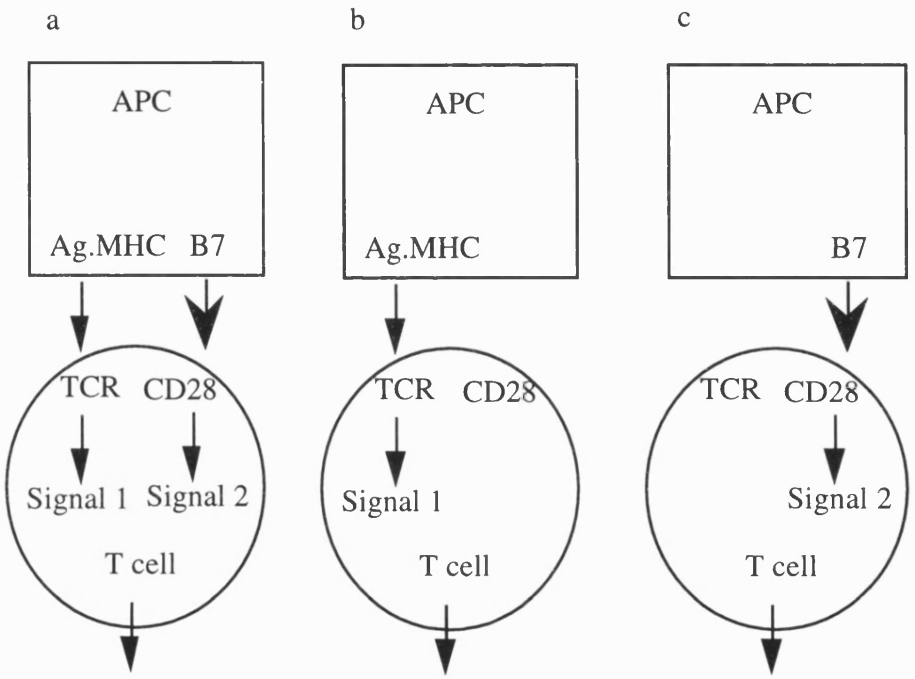


Fig. 1.3: Potential Outcomes of the Interaction between APCs and T cells

Individual APCs may supply antigen (ag) bound to MHC and / or CD80 (B7) or CD86 (B70) ligand. Encounter with resting T cells expressing TCR and B7 receptor, CD28 results in a) clonal expansion b) anergy or apoptosis c) no proliferative or anti-proliferative effect; depending on signals from the APC.

1.3 Biochemical Signalling through CD28

Unlike TCR derived pathways, very little is known about CD28 dependent events although it is accepted as a costimulatory molecule necessary and sufficient to induce IL2 gene expression when combined with TCR derived signals. However, there is no consensus view on how CD28 acts to costimulate a T cell, although a number of observations have been reported which may lead to the description of definitive pathway(s) arising from the activation of CD28, which combine with TCR pathways to activate T cells. Whether or not CD28 acts to synergize with the TCR or merely to amplify its signals is debatable, as they share a number of down stream signalling elements. For example, ligation of CD28 by αCD28 mAbs activated PLCγ1, with neither TCR ligation nor secondary crosslinking of CD28, leading to a rise in intracellular calcium levels (Nunes et al., 1993). On the other hand CD80 ligation of CD28 does not increase intracellular calcium or DAG levels (Ward et al., 1993). Consistent with characteristics of CD28 derived pathways, rather than TCR/ CD3 derived proximal effects, was the slower rate of PLCγ1 activation compared to its activation through CD3 (Nunes et al., 1993). CD3 ligation activated PLCγ1 maximally by ~30 seconds, while CD28 ligation took maximal effect after 60-120 seconds (Nunes et al., 1993). Thus, if under conditions of high aggregation CD28 does activate PLCγ1, it may be for the purposes of prolonging calcium-dependent pathways. However, the observation that CD28 could signal through calcium independent pathways was noted upon the treatment of the cells with the calcineurin inhibitor, cyclosporin A (CsA) whereby CD28 could still activate JNK (Su et al., 1994), NFkB (Lai and Tan, 1994) and $p70^{S6\ kinase}$ (S6K) (Pai et al., 1994) independent from the TCR.

TCR derived signals are sensitive to CsA due to inhibition of the phosphatase calcineurin which when activated dephosphorylates the cytoplasmic form of NFAT, an event required for nuclear entry of NFAT (Liu, 1993). Due to the CsA-sensitivity of TCR-derived NFAT nuclear translocation and T cell activation (Su et al., 1994; Lai and Tan, 1994; Pages et al., 1994; Kunz et al., 1993; Liu, 1993; Pai et al., 1994) and the insensitivity of phorbol 12-myristate 13-acetate (PMA) and CD28-stimulated IL2

secretion, it would appear that the target for CsA lies in the TCR pathway upstream of PKC (Kanoh et al., 1990; Linsley et al., 1991). This would indicate that while CD28 may share some of the same signalling elements as the TCR, it also has distinct effectors which are insensitive to inhibitors which affect TCR activated pathways.

The TCR and CD28 share a number of substrates which would be compatible with the coordinate induction of IL2 expression. Distally JNK (Su et al., 1994) and S6K (Pai et al., 1994) may be activated by both receptors. Proximally, the src family PTKs fyn and lck (Raab et al., 1995; Harlan et al., 1995; Mustelin, 1994; Boussiotis et al., 1996), Vav and Ras (Nunes et al., 1994), PLCγ1 (Nunes et al., 1993) and PI3K (Rudd et al., 1994) are associated with both receptors either functionally or physically. Two interpretations regarding the costimulatory role of CD28 are possible: either CD28 serves to amplify TCR derived signals to a threshold below which T cells would not display a response or different pathways activated through CD28 synergize with TCR derived signals to upregulate IL2 production (fig. 1.4). Both TCR and CD28 derived pathways contribute to PI3K activation and show additive activation of PI3K when both receptors are stimulated (Ward et al., 1993), although in another report it was suggested that the TCR did not activate PI3K and that the activation of PI3K was independent of PTKs e.g. fyn which were responsible for TCR proximal signalling events (Ward et al., 1992). The function of PI3K in CD28 signalling may be related to its homology with the yeast protein target of rapamycin (TOR) which is involved in sorting vacuolar and lysosomal proteins and G1 to S phase progression, the latter of which is induced in T cells by IL2 (Kunz et al., 1993). As discussed below (see section 1.5) acidic sphingomyelinase (ASMase), an enzyme involved in generating the potent second messenger ceramide, is localised in lysosomes. Therefore if yeast TOR and human PI3K have related function, CD28 may be able to modulate sphingolipid availability at a proximal stage through activation of PI3K. There are CD28 pathways which are distinct from the TCR, such as activation of ASMase (Boucher et al., 1995), a rapamycin-sensitive activation of S6K, CsA insensitive IL2 production (Price et al., 1992; Kunz et al., 1993) and the tyrosine phosphorylation of distinct substrates (Hutchcroft and Bierer, 1994). Ligated CD28 affects pathways separate from and shared with the TCR.

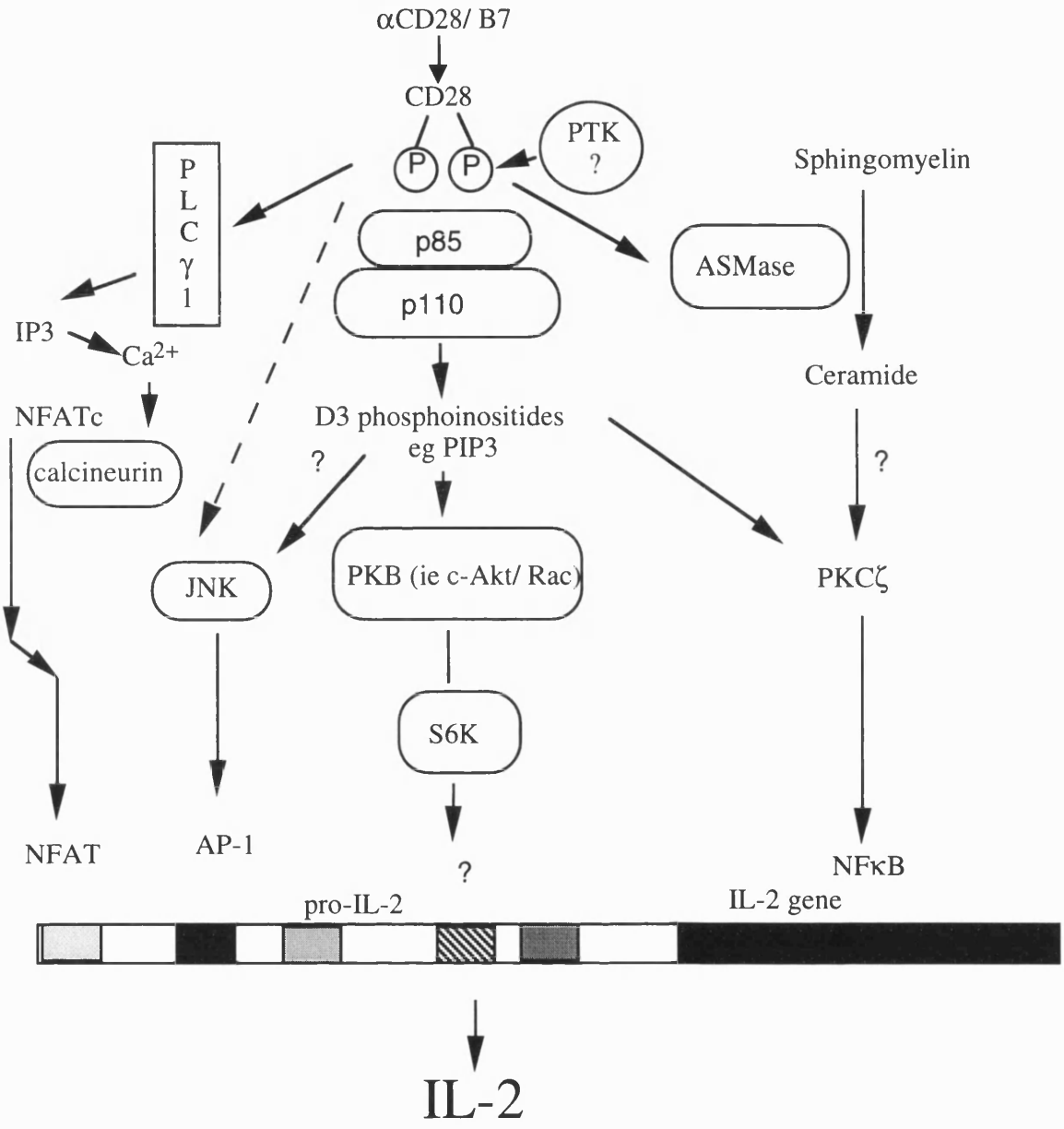


Fig. 1.4: CD28 Signalling Pathways

CD28 is involved in many pathways and is necessary for full T cell activation. It induces a number of transcription factors which cause the upregulation of IL2, a cytokine with pleiotropic paracrine and autocrine effects. ?, proposed activation pathways; dashed arrows, multiple step pathways; solid arrows, cited direct association and/ or activation within pathways; pro-IL-2, IL-2 promoter.

It has not been determined whether the regulation of TCR/ CD28-shared substrates or the modulation of CD28-specific effectors has the greater contribution to CD28 costimulatory effects.

1.31 The Cytoplasmic Domain of CD28

The intracellular domain of CD28 is 40 amino acids long and in single letter coding reads:

```
180         190         200         210         220
 |           |           |           |           |
RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS
```

Putative SH2 docking sites are indicated in heavy type and putative PKC phosphorylation sites are underlined. The cytoplasmic domain of CD28 bears the amino acid sequence YMNM (residues 191-194) which when tyrosine phosphorylated matches the reported consensus sequence requirement for binding p85, the regulatory subunit of PI3K. Like the binding of ZAP70 to ITAMs (Hatada et al., 1995), this occurs through SH2 domains, although the SH2 domains of p85 have the preferred sequence pY(M/V/I/E)XM where X is any amino acid (Songyang et al., 1993). Between ligation of CD28 and recruitment of PI3K, the SH2 binding site of CD28 must be tyrosine phosphorylated (Lu et al., 1994; Ueda et al., 1995; Stein et al., 1994; Raab et al., 1995; Ward et al., 1995; Prasad et al., 1994; Pages et al., 1994; August and Dupont, 1994b). As CD28 has no intrinsic kinase activity, a cytosolic or membrane associated kinase must perform this function. The identity of such kinase(s) phosphorylating CD28 following ligation remains unknown although the necessity of src family kinases in CD28 derived costimulatory pathways has been suggested through the reputedly selective src kinase inhibitor, herbimycin A (Vandenberghe et al., 1992). The proximal role of PTKs in TCR signalling and the involvement of PTKs in CD28 signalling was suggested by the ability of PMA (a DAG mimic and thus PKC activator) and ionomycin (a calcium ionophore) to increase IL2 expression and secretion in a herbimycin A-insensitive manner. However cells treated with PMA after CD28 crosslinking showed a decrease in IL2 production and a 100 kDa substrate was no longer tyrosine phosphorylated after the addition of herbimycin A

(Vandenberghe et al., 1992). Herbimycin A markedly inhibits CD28-PI3K association (Lu et al., 1994) indicating a significant role for *src* family kinases in the CD28 transduction pathway. However, PI3K recruitment to CD28 was not completely inhibited and one group reported that herbimycin A had no effect on CD28 signalling (Stein et al., 1994), although neither their data nor analysis parameters were discussed. Thus the necessity of PTKs in CD28 signalling remains contentious.

Another tyrosine kinase $p72^{itk/EMT}$, a member of the Tec family of PTKs, associates with CD28 (King et al., 1997) becoming activated after CD28 ligation (King et al., 1997; Gibson et al., 1998). Tec PTKs possess an extensive amino terminus, an SH2, SH3 and kinase domain although they lack the negative regulatory tyrosine residue common to src PTKs (August et al., 1994). The SH2 and SH3 domains likely provide a mechanism by which itk associates itself with CD28 and so becomes activated. Peptides representing the diproline motifs on the cytoplasmic tail of CD28 when mutated P196A or P208A revealed a greater reliance on the amino terminus proline P196 in order to activate itk (Marengere et al., 1997). Studies on itk activation through SH2 interactions are less clear whereby one study which demonstrated a partial reliance on Y191 to activate itk also demonstrated in the same study that mutation of all four tyrosine residues on the cytoplasmic tail of CD28 augmented itk activation above control wild type levels of activation (King et al., 1997). A separate study also indicated Y191 contributed to itk activation, although in contrast to the previous report (King et al., 1997), mutation of four tyrosine residues ablated itk activation (Gibson et al., 1998) possibly due to the use of Jurkats transfected with murine CD28 rather than a chimaeric protein composed of CD8 extracellular and transmembrane domains coupled to CD28 intracellular domain in the study of King (King et al., 1997). Similarly contradictory data exists on the direct or indirect nature of itk activation following CD28 ligation. In one report a direct activation of itk by CD28 was suggested by the rapidity of itk activation following CD28 ligation and because itk was activated without CD28 crosslinking and preceded lck activation (August et al., 1994). Other reports indicated that itk could not phosphorylate CD28 in the absence of lck (Raab et al., 1995; Gibson et al., 1996) nor was it activated in an lck -ve cell line, JCaM1 (Gibson et al., 1998). An SH3 mediated interaction between itk and

src PTKs may be suggested as it has been found that a proline rich motif on itk, KKPLPPTPED, associates with the SH3 domain of fyn (Cheng et al., 1994). While the mechanism by which itk is activated remains to be firmly established, itk has been demonstrated to phosphorylate all four tyrosine residues on the intracellular domain of CD28 while lck appeared to phosphorylate only Y191 (King et al., 1997). Furthermore the role of itk in T cell biology may be one of negative regulation because a three fold increase in proliferation of CD3, CD28 stimulated murine $itk^{-/-}$ T cells was observed over murine $itk^{+/-}$ T cells (Liao et al., 1997).

Interestingly, CTLA4 , the CD28 homologue, also has the consensus motif for binding p85, although the particular sequence is YVKM (June et al., 1994). There are conflicting data on whether this sequence on CTLA4 , when phosphorylated can recruit PI3K in a T cell. In one report which details an association between CTLA4 and PI3K there was unfortunately no comparison against CD28 (Schneider et al., 1995b), although the production of D-3 lipids associated with CTLA4 immunoprecipitates compared to p85 immunoprecipitates was small in comparison to the level of D-3 phospholipids associated with CD28 observed in other studies (Pages et al., 1996; Stein et al., 1994; Ward et al., 1995). In another study, contrary to the results of Schneider, chimaeric constructs comparing the contribution of CD28 and CTLA4 cytoplasmic domains in the stimulation of IL2 secretion, revealed that CD28 but not CTLA4, bound and activated PI3K and stimulated IL2 gene expression (Stein et al., 1994). Therefore it appears likely that PI3K has a more significant role in CD28 signalling rather than that of CTLA4.

The intracellular domain of CD28 has three possible phosphorylation sites for PKC, namely the sequences SKR, TPR and TRK. Studies on the effect of PMA on the phosphorylation states of CD28 revealed that PMA stimulation of Jurkat T cells lead to threonine phosphorylation of CD28 and αCD28 mAb ligation of CD28 lead to tyrosine phosphorylation (Hutchcroft et al., 1996) and that PMA and CD28 signals together decreased the level of tyrosine phosphorylation observed (Hutchcroft et al., 1996). However the large increase in tyrosine phosphorylation observed may have been an

artefact of antibody ligation because CD80 stimulation of CD28 increased phosphorylation of tyrosine residues marginally and serine or threonine residues much more (Parry et al., 1997). Significantly PMA stimulation of Jurkats in conjunction with CD80 stimulation of CD28 decreased both the recruitment and activation of PI3K, a signalling molecule believed to be important in cell growth (Parry et al., 1997). Inhibition of PKC or PI3K using the inhibitors Ro-31 and wortmannin respectively did not affect the CD80-induced phosphorylation of CD28 (Parry et al., 1997) suggesting other kinases are involved in phosphorylation of CD28.

1.32 Phosphatidylinositol 3-Kinase; a Key Effector in CD28 Signalling

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase which phosphorylates phospholipids on the D3 position of the inositol ring resulting in the generation of phosphatidylinositol $_3$ phosphate (PI_3P), phosphatidylinositol $_{3,4}$ bisphosphate ($PI_{3,4}P_2$) and phosphatidylinositol $_{3,4,5}$ trisphosphate ($PI_{3,4,5}P_3$). The function of these are not known although the physical association of PI3K and CD28 has been the subject of intense scrutiny. Various groups have shown an association between the cytoplasmic domain of CD28 and PI3K (Lu et al., 1994; Ueda et al., 1995; Stein et al., 1994; Raab et al., 1995; Ward et al., 1995; Prasad et al., 1994; Pages et al., 1994; August and Dupont, 1994b) leading to PI3K activation and D3-phospholipids are believed to be important second messengers (Ueda et al., 1995).

PI3K may be activated *in vitro* by a tyrosine phosphorylated peptide derived from the platelet derived growth factor receptor (PDGFR)β SH2 docking domain and other molecules involved in proliferation of cells (see table 1.1) (Rudd et al., 1994; June et al., 1994). The interaction between PI3K and receptors is based upon p85, the regulatory subunit of PI3K. p85 has two SH2 domains which when bound to the tyrosine phosphorylated sequences on a receptor are predicted to form a coiled coil between the SH2 motifs, necessary and sufficient for the interaction of p85 with p110, the catalytic subunit of PI3K (Hatada et al., 1995). The domain between the two SH2 sites, as suggested for ZAP70 (Hatada et al., 1995), might also interact with tyrosine kinases e.g. lck and fyn. Therefore PTKs may facilitate the activation of PI3K by tyrosine

phosphorylation of the SH2 binding sites of p85 and/ or interacting with the domain between the SH2 regions. In fact PDGFR, EGFR (epidermal growth factor receptor), TCR, IL-2R, IL-4R and IL-7R (interleukin-2, -4 and -7 receptors) when ligated can activate PI3K to varying degrees (Rudd et al., 1994; June et al., 1994). As so many growth factor receptors recruit and activate PI3K, a role for PI3K in proliferation would seem likely after recruitment to CD28. However the effects of PI3K activation depend upon the cell type analyzed as demonstrated by the fungal metabolite, wortmannin, a specific inhibitor of PI3K at nanomolar concentrations (Ward et al., 1995).

Protein	Sequence
CD28	YMNM
PDGFRβ	YMDM
CSF-1R	YVEM
cKIT	YMDM
polyoma MT	YMPM
IRS-1	YMNM
IL-7R	YVTM
CTLA-4	YVKM

Table 1.1: The Consensus SH2 Binding Sequence of PI3K Is Shared by Many Receptors

PDGFRβ, platelet derived growth factor receptor β chain; CSF-1, colony stimulating factor -1; MT middle T antigen; IRS-1, insulin receptor substrate -1; IL-7R, interleukin-7 receptor; CTLA4, cytotoxic T cell associated antigen-4.

- - -

The irreversible binding of wortmannin to the ATP binding site on the catalytic subunit p110 of PI3K does not prevent CD28-PI3K association (Ward et al., 1995), but reduces IL2 production in resting T cells while in the leukaemic T cell line, Jurkat, it increases IL2 production (Ueda et al., 1995). In neurons wortmannin demonstrated an anti-

apoptotic effect of PI3K (Yao and Cooper, 1995) following ligation of the nerve growth factor receptor. Thus PI3K appears to be involved in positive regulation of proliferation in resting T cells, negative modulation of proliferation in Jurkats and have an anti-apoptotic effect in neurons.

In T cell activation therefore there appears to be a stage specific change in the role of PI3K. This may be caused by an alteration in the binding preference or availability of PI3K from CD28 to CTLA4 as a T cell becomes activated. Some data suggest that the binding site Y191 of PI3K on CD28 when mutated to Y191F in order to ablate PI3K binding to CD28, also causes a sustained expression of CD28 on the cell surface (Cefai et al., 1998). As such a mutant has previously been shown to prevent costimulation (Cai et al., 1995), it is possible that internalization of CD28 is necessary for signal transduction i.e. by preventing internalization of CD28 through disruption of the PI3K binding site, CD28 may be prevented from reaching its signal transduction site. Paradoxically sustained surface expression of CD28 may facilitate CD28 signalling by creating greater opportunity for CD28-CD80/ CD86 interaction. Currently it is not known whether CD28 internalization is necessary for CD28 signal transduction. It was demonstrated that complexes of CD28 and PI3K associated with clathrin associated AP2 complexes and that 50% of internalized CD28 was directed toward lysosomes, while 50% was recycled to the cell surface (Cefai et al., 1998). Interestingly the mutant Y209F showed twice as much CD28 internalization as wild type CD28 indicating that Y209 may play a role in sustaining CD28 surface expression. Additionally the mutant Y165F of the PI3K binding site YVKM on CTLA4 showed enhanced surface expression (Leung et al., 1995) similar to mutation of the CD28 PI3K binding site (Cefai et al., 1998). It would therefore imply that both receptors may be internalized by a similar mechanism. However while CTLA4 associates with the μ2 subunit of AP2, CD28 does not (Shiratori et al., 1997). Furthermore the intracellular domain of CTLA4 does not possess a consensus PKC phosphorylation site adjacent to the PI3K binding site unlike CD28 and these differences may account for the delay in CTLA4 surface expression. Indeed PMA can inhibit the association of CD28 and PI3K (Hutchcroft et al., 1995), the significance of this may possibly be that in a costimulated T cell transduction cascade, PI3K initially

associates with CD28 whereby CD28 is internalized. The ligation of the TCR may ultimately lead to CD28 phosphorylation through PKC activation in a manner analogous to incubation of T cells with PMA, a known PKC activator (Vance and Vance, 1991). The putative phosphorylation of CD28 by PKC may prevent further association of PI3K with CD28 and so PI3K may be recruited to an alternative receptor, namely CTLA4, initiating negative regulation of T cells.

1.33 CD28 and Cell Cycling

One response observed upon T cell costimulation is a large population expansion of the antigen-APC-responsive T cells. Cells in a resting state may be attributed to being in G1/ Go of the cell cycle. In overcoming the cell cycle entry checkpoint, whereby a cell may either stop cycling and enter Go or pass from G1 to S phase, a variety of inhibitory and stimulatory factors are thought to be modulated (Alberts et al., 1989). The nature of these signals remains obscure in T cells (Smith, 1984). CD28 may be involved in facilitating the passage of resting T cells to cycling stages through signalling intermediates which finally stimulate S6K, an enzyme which promotes cell cycle progression through phosphorylation of S6, a ribosomal processing protein (Pai et al., 1994). PI3K activation (Burgering and Coffer, 1995; Franke et al., 1995) and so production of PI_3P (Franke et al., 1995) was demonstrated to be sufficient to activate protein kinase B (PKB=Akt=RAC), a serine/ threonine kinase, itself activated by phosphorylation on serine. The extent of PKB activation was four fold greater following stimulation by PI_3P than by the unphosphorylated precursor (Franke et al., 1995). Subsequently, activated PKB phosphorylated and activated S6K (Burgering and Coffer, 1995). S6K, like PKB is a serine/ threonine kinase and to become activated is phosphorylated on multiple serine/ threonine residues (Price et al., 1992). In turn it phosphorylates the 40S ribosomal subunit protein S6, which was proposed not to activate T cells, but to initiate cell cycle progression from G1 to S phase (Pai et al., 1994). This may then be the trigger by which resting T cells overcome the restriction point in G1 to enter the proliferative phase of cell cycling (Alberts et al., 1989) and a mechanism for CD28 to effect cell cycling.

The molecule rapamycin inhibits CD28 but not TCR derived pathways (Su et al., 1994; Lai and Tan, 1994; Sabatini et al., 1994; Price et al., 1992; Pai et al., 1994). While it is accepted that CD28 ligation leads to recruitment and activation of PI3K, evidence of its involvement in S6K activation was circumstantial until it was shown that crosslinking CD28 alone was sufficient to cause S6K activation (Pai et al., 1994). While CD28 (or IL2 (Kunz et al., 1993))-derived activation of S6K could be inhibited by rapamycin, TCR-derived activation of S6K was rapamycin insensitive. The independence of CD28 from the calcium pathway was confirmed by an insensitivity to CsA, while the TCR could not activate S6K after CsA treatment (Franke et al., 1995; Price et al., 1992). Although S6K could not be activated through a CD28 derived pathway in the presence of rapamycin, the upstream activator PKB was insensitive to rapamycin, showing rapamycin to have a distal target in the CD28 signalling pathway. These data support the existence of separate signalling pathways in CD28 and TCR signal transduction which ultimately may impinge on shared substrates.

1.4 CD28 Proximal and Distal Signals

The secretion of IL2 from T cells requires the translocation (from the cytosol to the nucleus) or induction of transcription factors which regulate IL2 promoter activity by association with their respective binding sites. The transcription factors involved in the stimulation of the IL2 gene promoter include the nuclear factor of activated T cells (NFAT), activation protein-1 (AP1), activation protein-3 (AP-3), OCT-1 and nuclear factor κB (NFkB) (Granelli-Piperno and Nolan, 1991). The contribution each of these makes to upregulation of the IL2 expression is unclear due to different methods and origins of T cells used to assess the impact of various mitogenic, primary and costimulatory signals (Umlauf et al., 1993) used to stimulate T cells to produce IL2. Of the data available the contribution CD28 and the TCR make to induce NFAT, NFkB and AP1 is summarised in figure 1.5.

1.41 NFAT

The primary stimulus for T cell activation derived from ligation of the TCR/ CD3 results in the dephosphorylation of a cytoplasmically located transcription factor, NFAT, which is involved in IL2 promoter activation (Granelli-Piperno and Nolan, 1991). When NFAT is dephosphorylated by the calcium-dependent phosphatase calcineurin it may translocate to the nucleus. Ligation of the TCR results in an increase in $Ca^{2+}i$ (Smazel and Resch, 1995) and ultimately translocation of NFAT to the nucleus which may be inhibited by cyclosporin A (CsA) (Liu, 1993). CsA binds and inhibits the cis-trans isomerase activity of cyclophilin and this complexes with calcineurin preventing activation of calcineurin. Interestingly CD28 which is insensitive to CsA inhibition (Su et al., 1994; Lai and Tan, 1994; Pages et al., 1994; Kunz et al., 1993; Liu, 1993; Pai et al., 1994), surprisingly may also activate PLCγ (Nunes et al., 1993). Activation of PLCγ following TCR ligation generates the second messengers IP_3 and DAG from $PI_{4,5}P2$. IP_3 causes a rise in intracellular calcium levels and this leads to activation of the phosphatase calcineurin (Smazel and Resch, 1995). However as only saturating concentrations of αCD28 mAb activate PLCγ (Nunes et al., 1993) it is doubtful whether or not CD28 *in vivo* i.e. under stimulation from the B7 family of ligands, is capable of activating calcineurin or

dephosphorylating NFATc allowing its subsequent translocation to the nucleus. The consensus view is that NFAT translocation is controlled by a TCR-stimulated signalling pathway.

1.42 NFkB

NFkB is a member of the Rel family of transcription factors. This family comprises the elements c-Rel, Rel A, Rel B, NFKB1 (p50 and precursor p105) and NFKB2 (p52 and precursor p100). NFKB1 p105 and NFKB2 p100 which sequester Rel family proteins, as IκBα does, were not down regulated by CD28 ligation, unlike IκBα (Λαι αvδ Ταv, 1994). Increased levels of c-Rel were noted in the nucleus in the presence of CD28, PMA signals (Lai and Tan, 1994), although not with CD28 signals alone in resting T cells (Bryan et al., 1996). However another study showed CD80 could stimulate NFkB generation without additional signals in T cell blasts (Edmead et al., 1996). The mechanism of IκBα down-regulation by CD28 may be through PKCζ which can phosphorylate IκBα facilitating NFkB activation (Lozano et al., 1994). PKCζ , an atypical PKC isoform which is insensitive to activation by phorbol ester and calcium, may be activated by $PI_{3,4,5}P3$, a PI3K product (Nakanishi et al., 1993). This may be the mechanism by which CD28 contributes to the activation of NFkB, a transcription factor involved in IL2 gene upregulation.

An inhibitor of CD28 signals, rapamycin, demonstrated the involvement of CD28 in inducing the translocation of the Rel family member, c-Rel to the nucleus (Lai and Tan, 1994). There may be more than one target for rapamycin (it impinges on both S6K activation and NFkB induction) including RAFT1 or a mammalian homologue of the yeast TOR (target of rapamycin) proteins (Pai et al., 1994; Kunz et al., 1993). Alternatively a single target of rapamycin may impinge on more than one signalling pathway. This would lie downstream of PKB and upstream of IκBα, in which case the PI3K to PKB signal cascade may play a role in induction of NFκB.

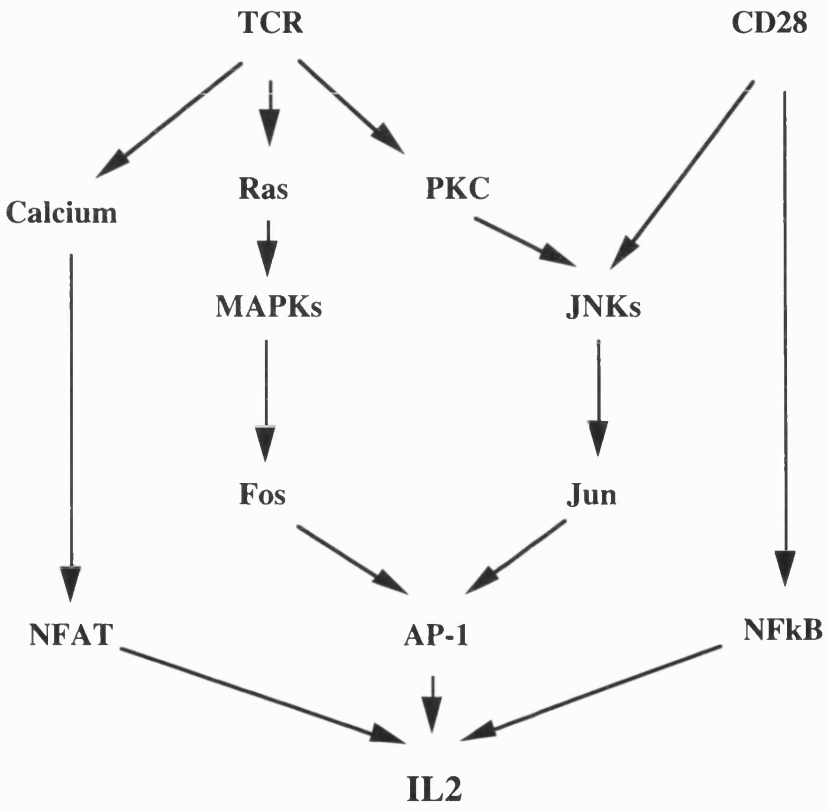


Fig. 1.5: Contributions of CD28 and the TCR to IL2 Expression

CD28 and the TCR differentially regulate signals inducing binding of the transcription factors NFAT, AP1 and NFkB to the IL2 promoter, required for IL2 secretion.

1.43 AP1

Another transcription factor required for IL2 transcription is AP1, which is formed from a complex of c-Fos and c-Jun proteins (Su et al., 1994). Induction of c-Fos is associated with TCR derived signals initiated after activation of the small GTPase Ras. Ras, a cytoplasmic GTP-binding protein with intrinsic GTPase activity is active in its GTP-bound form. The level of active Ras is regulated by a dynamic equilibrium in the exchange of GDP for GTP bound to Ras, whereby GTPase activating proteins negatively regulate Ras and guanine nucleotide exchange factors such as Sos (son of sevenless) and Vav promote Ras activation (Smazel and Resch, 1995). Association of Ras with the TCR, through a complex of the adaptor proteins SHC (src homology and collagen adapter protein), GRB2 (growth-factor receptor bound-protein 2) and SOS facilitates the exchange of GDP to GTP on Ras (Rudd et al., 1994). Interestingly antibody ligation of CD28 has been demonstrated to activate Ras but this effect was not observed by CD80 ligation of CD28 (Nunes et al., 1994). Therefore, like the translocation of NFAT, c-Fos would appear to be sensitive to TCR, but not CD28, signals. One of the functions of Ras is to stimulate the MAPK cascade (fig. 1.6). After ERK1 and ERK2 activation, the transcription factor Elk1 is induced to bind to a *Fos* promoter, upregulating Fos expression. The activation of ERK1 and ERK2 occurs in a CD28- and calcium-independent fashion i.e. are PKC-dependent although their activation alone showed no correlation with IL-2 production (Su et al., 1994).

AP1 formation also requires another component, c-Jun (Su et al., 1994; Granelli-Piperno and Nolan, 1991). JNK, a novel member of the MAP kinase group, phosphorylates and augments the transcriptional activity of c-Jun and possibly JunD (Su et al., 1994). PMA and calcium ionophore (Ionomycin) stimuli or alternatively costimulation of the TCR and CD28 lead to full activation of JNK (Su et al., 1994). Alone each stimulus resulted in little or no activation. The calcineurin inhibitor, CsA, inhibited the synergistic activation of JNK. Thus at least at one level, an integration of TCR and CD28 signals occurs, leading to induction of nuclear transcription factors which can bind to the IL-2 promoter (Su et al., 1994). MAP kinase subfamily members ERK and JNK, when phosphorylated and activated, translocate to the nucleus and so it is

possible that CD28 and TCR derived signals synergize not only in the cytosol at JNK, but also in the induction of composite transcription factors, leading to T cell activation (Su et al., 1994). As the activation of JNK and IL2 production in the presence of PMA and ionomycin is further increased by CD28 ligation (Su et al., 1994), full T cell activation could be attributed to at least three distinct pathways involving calcium, PKC and CD28 which may be interdependent. While JNK is a distal cytosolic signalling element and the intermediate steps between CD28 ligation and JNK activation have yet to be found, PI3K has been proposed to regulate the MAP kinase cascade through $PI_{3,4,5}P3$ (Ward et al., 1995) (fig. 1.6). A complex cascade of events between ligation of the tyrosine kinase receptor, EGFR, and JNK activation has been shown to involve a number of small GTPases which are members of the Ras superfamily. The Rho subfamily GTPases Rac1, 2 and Cdc42Hs, but not RhoA, were shown to activate proximal signalling elements leading to the activation of JNK (Minden et al., 1995). As CD28 has been previously shown to interact with Ras when crosslinked (Nunes et al., 1994), it is possible that a CD28-Ras association may stimulate the induction of AP1 , a transcription factor necessary for IL2 expression (Granelli-Piperno and Nolan, 1991). However while antibody ligation of CD28 may lead to Ras activation, ligation by the ligand CD80 was found to be insufficient to activate Ras (Nunes et al., 1994). Therefore it would appear more likely that CD28 contributes to the activation of JNK through a Ras-independent mechanism. As $PI_{3,4,5}P_3$ has been demonstrated to be necessary for Rac activation (Rodriguez-Viciana et al., 1994) and Rac stimulates the activation of JNK (Minden et al., 1995), it is possible that CD80 ligation of CD28, shown to stimulate PI3K activation and recruitment to CD28 (Ward et al., 1995), provides a means by which CD28 contributes to AP1 formation.

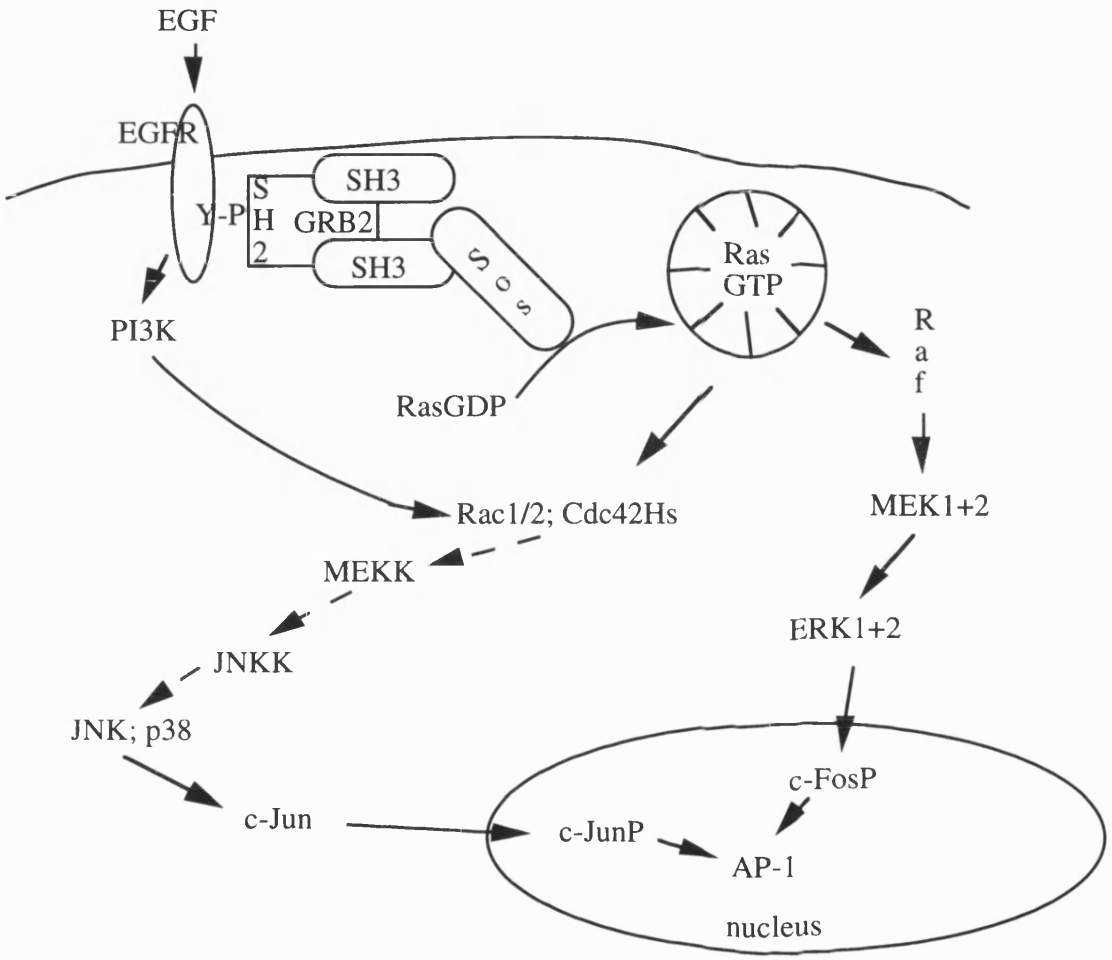


Fig. 1.6: Small GTPases Rac 1 and 2 and Possibly Cdc42Hs are Involved in Activation of JNK.

Epidermal growth factor receptor (EGFR) associates with a GRB2, SOS complex and activates Ras. Subsequently MAP kinases and JNK are activated leading to transcription factors becoming phosphorylated and translocating to the nucleus. CD28 also associates with GRB2.SOS and can activate Ras upon crosslinking.

1.5 Sphingomyelin and Ceramide

Sphingomyelin is a ubiquitous animal membrane component comprising a hydrophobic ceramide moiety which contains a mixture of fatty acids amide linked to sphingosine or other related long chain aliphatic amines (sphingoid bases) and a hydrophilic polar head (phosphocholine) (see fig. 1.7 below). Sphingomyelin may be synthesised in microsomes by cytidyl diphosphate (CDP) choline: ceramide phosphocholine transferase which catalyzes the transfer of phosphocholine from CDP-choline to ceramide to form sphingomyelin (Vance and Vance, 1991).

Sphingomyelin is catabolized by two sphingomyelinases (SMases). One of these has a neutral pH optimum (referred to as NSMase) and is membrane bound, the other is lysosomal and has an acidic pH optimum (referred to as ASMase) (Wiegmann et al., 1994). The breakdown of sphingomyelin produces ceramide and phosphocholine. The latter of these two products is recycled in the CDP-pathway upon combination with choline to become CDP-choline. Choline phosphotransferase may combine CDP-choline with DAG to generate phosphatidylcholine (Vance and Vance, 1991). Sphingolipid metabolites such as sphingosine, sphingosine-1-phosphate and ceramide have other functions aside from being intermediaries in sphingolipid metabolism and these are discussed below.

The ceramide moiety embeds itself in hydrophobic diacylglyceryl chains of phospholipids and other membrane lipids (Vance and Vance, 1991). Sphingomyelin is distributed in intracellular and plasma membranes. In the latter it is symmetrically distributed. However carbohydrate-containing sphingolipids (glycosphingolipids) are predominantly found in the plasma membrane, asymmetrically distributed such that the polar head group extends toward the extracellular environment (Vance and Vance, 1991).

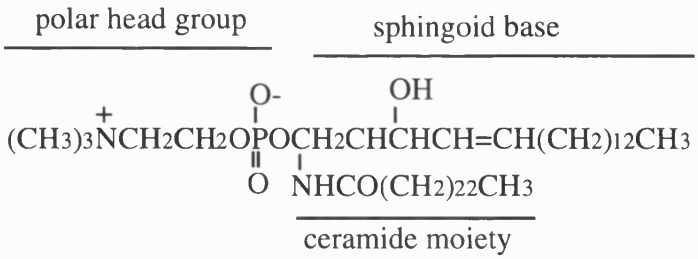


Fig. 1.7: Moieties of Sphingomyelin

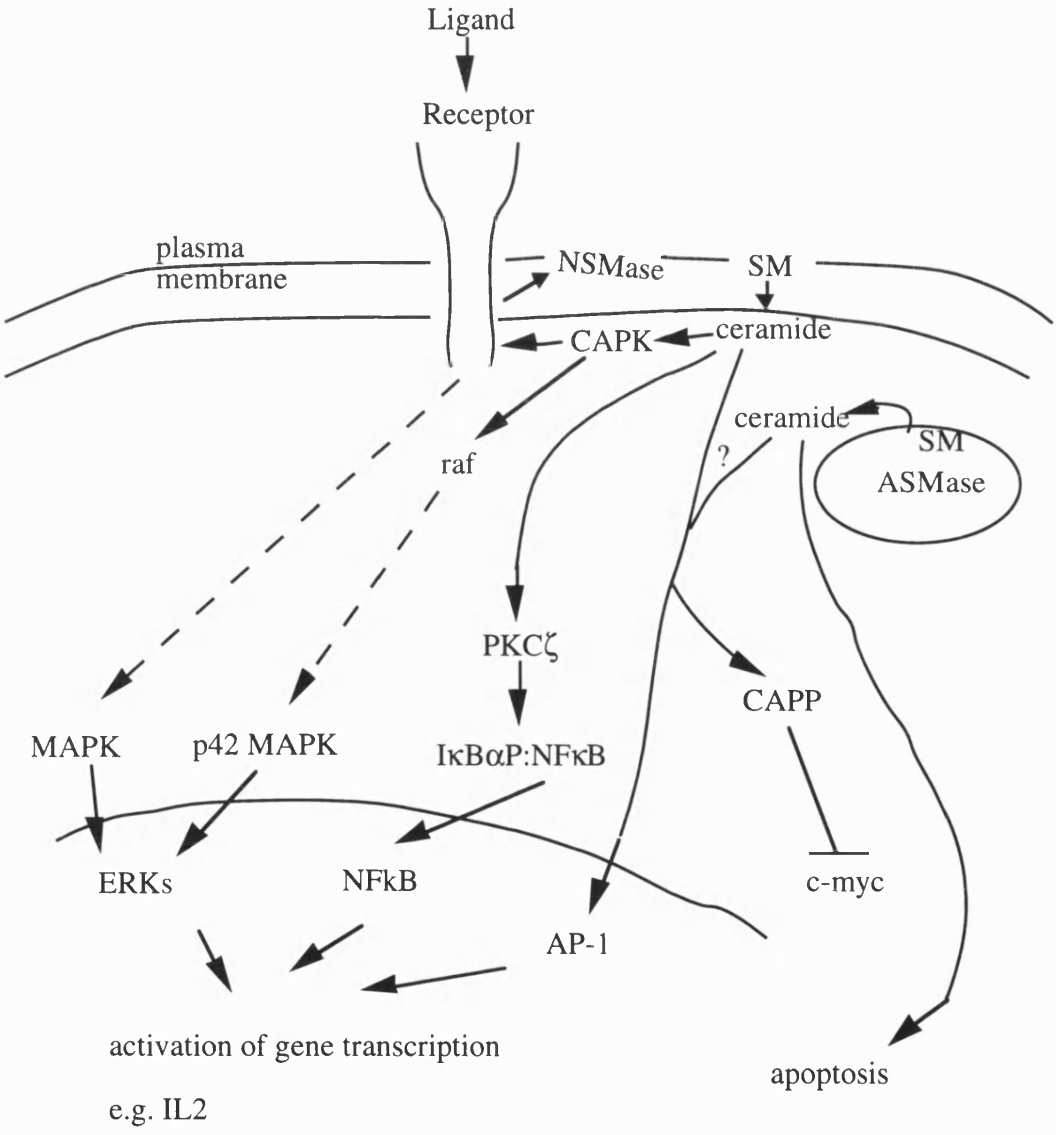


Fig. 1.8: Function and Targets of Ceramide in Cell Signalling

Ceramide has many targets and effects. Dashed arrow indicates multistep pathway; ? indicates that source of ceramide in vivo unclear i.e. could be N- or A-SMase derived; solid arrows indicate published effects.

1.51 Ceramide

Ceramide is reported to have many targets as discussed below, as well as being a precursor for glycosphingolipids, which have modulatory function on transmembrane signal transduction (Felding-Habermann et al., 1990; Hakomori, 1990). Different forms of ceramide exist which are made from the combination of a sphingoid base being acylated by various fatty acids. The nomenclature of ceramides often refers to the number of carbon atoms in the acyl chain. For example when 2 carbons are present in the acyl moiety, the molecule would be referred to as C2 ceramide. Different versions of ceramide present the possibility of different function amongst ceramide molecules (Vance and Vance, 1991), although in murine splenocytes both C2 and C6 ceramide costimulated proliferation (Boucher et al., 1995; Chan and Ochi, 1995). A comparison of C6 and C16 ceramide on c-Raf activation revealed a quantitative difference in effect between the two types of ceramide in that c-Raf was less sensitive to activation by C6 ceramide *in vivo* whereas *in vitro* c-Raf could be activated comparably by either molecule (Huwiler et al., 1996). The lower *in vivo* activity associated with the shorter chain length C6 ceramide may be due to reduced uptake of shorter ceramides as opposed to sphingoid molecules with a longer chain (Merrill et al., 1989). In this thesis where a particular species of ceramide has been reported reference is duly made, otherwise a generic term, ceramide is employed to describe acylated sphingoid bases ie ceramides. Targets of ceramide include a 97 kDa proline directed serine/ threonine ceramide activated protein kinase (CAPK) (Mathias et al., 1993; Liu et al., 1994). This is membrane bound, activated by ceramide after TNFR and IL-1R stimulation (Mathias et al., 1991; Mathias et al., 1993) and has been shown to activate Raf (Liu et al., 1994), implicating ceramide as a proximal effector of the mitogen activated protein kinase (MAPK) cascade. As repeated stimulation of the TCR can induce AICD (Van Parijs et al., 1996), it is possible that CAPK activation may lead to negative regulation of T cell viability. Activation of a cytosolic ser/ thr protein phosphatase, CAPP (Dobrowsky and Hannun, 1992), PKCζ (Lozano et al., 1994), p42 MAPK (Raines et al., 1993), and JNK (Kyriakis et al., 1994) as well as downregulation of c-Myc (Kim et al., 1991; Wolff et al., 1994), phosphorylation and inactivation of IkB (Machleidt et al., 1994) and nuclear translocation of NFκB (Schutze et al., 1992; Yang et

al., 1993; Johns et al., 1994) and AP1 (Johns et al., 1994) have been described in different systems as being attributable to ceramide.

In one study involving ceramide generation following TNFR1 (55kDa) stimulation, it was found that both neutral and acidic SMases were activated independently by different cytoplasmic domains of TNFR1 and each type of SMase was coupled to distinct pathways. Neutral (N)SMase-derived ceramide activated a proline-directed kinase, CAPK and a phosphatase, CAPP (a phosphatase (PP) 2A) while ASMase-generated ceramide caused the translocation and activation of NFkB (Wiegmann et al., 1994). Significantly there was no crosstalk between the two pathways. Further ASMase but not NSMase activation was stimulated by PC-PLC-generated DAG (as shown by the PC-PLC specific inhibitor, D609). The region of TNFR1 responsible for activation of both PC-PLC and ASMase also contains an amino acid motif referred to as a death domain, believed to be necessary for TNFR to transduce apoptotic signals (Schutze et al., 1992; Cifone et al., 1993). As ceramide is widely implicated in facilitating apoptotic pathways, this finding may be more than coincidental .

A contrary finding on the separation of function between NSMase and ASMase, whereby both could stimulate NFkB translocation (Schutze et al., 1992), may be explained by the experimental procedure. When either permeabilized or homogenised cells are used, mixing of lysosomal and plasma membrane SMases may occur. Therefore where N- and ASMase activities are mixed together it would be difficult to determine whether A/NSMase or both activities were responsible for NFkB activation. However as pH optima were adjusted for neutral and acidic conditions, it does appear that neutral as well as acidic SMase can induce transcription factor translocation (Schutze et al., 1992). The fate of exogenous SMase in cell culture has not been reported but as there is much sphingomyelin in the plasma membrane and cellular NSMase is reputed to be located on the outer leaflet of the plasma membrane (Das et al., 1984), hydrolysis of sphingomyelin by exogenously applied SMase may provide a route for it to associate/ embed itself within the cell.

Ceramide can inhibit mitogenically stimulated splenic T cell growth (Felding-Habermann et al., 1990) and that of Molt4 leukaemic cells (Jayadev et al., 1995) and one possible effector of this function of ceramide may be CAPP (Dobrowsky and Hannun, 1992; Dobrowsky et al., 1993). While low concentrations of ceramide activate CAPP (maximum activation at 20μM) (Dobrowsky et al., 1993), low concentrations of okadaic acid (IC_{50} of less than 1nM) inhibit a ceramide-induced activation of CAPP (Dobrowsky and Hannun, 1992). Similar concentrations of okadaic acid (Wolff et al., 1994) also attenuated a ceramide-induced down-regulation of c-Myc mRNA levels (Kim et al., 1991). The retinoblastoma gene product Rb, in its dephosphorylated form, has been implicated in c-Myc down-regulation and in inhibiting cell cycle progression (Cooper and Whyte, 1989). Thus the inactive phosphorylated form of Rb, may present a substrate for CAPP, facilitating the role of ceramide in inhibiting cell growth and cell differentiation. Indeed C6 ceramide can dephosphorylate RbP and cause MOLT-4 cell cycle arrest. In cells lacking Rb, C6 ceramide could not induce cell cycle arrest indicating that ceramide may effect cell cycle arrest through dephosphorylation of RbP (Dbaibo et al., 1995).

With ceramide appearing to have so many targets, is it possible to draw a hypothesis on its mode of action in any one cell? In a simple model one could examine the paradox of ceramide having negative outcome on cell viability i.e. driving apoptosis (Cifone et al., 1993) and positive function i.e. mitogenesis and activation of cells (Mathias et al., 1993). As two or more SMases exist with distinct cellular locations and apparently discrete signalling pathways, it is debatable whether or not additions of ceramide to cultures or preparations, acts on each pathway equally or shows a preference for one more than another. Low concentrations of C2 ceramide (e.g. 0.03μM N-acetyl-sphingosine) showed a dependence on additional signals from TNFR1 ligation to induce HL-60 cell differentiation to a monocytic phenotype. At 6μM C2 ceramide could independently cause differentiation and inhibited cell growth by 57% with little or no effect on cell viability (85% viability by day 3 of culture) (Kim et al., 1991). Thus altering the concentration of exogenously added C2 ceramide changes the effect of C2 ceramide from that of differentiation to inhibition.

Figure 1.8 summarises the involvement of ceramide in cell signalling. Much of these data were obtained from studies using different cell types and through stimulation of different receptors and so a generic receptor has been shown. It appears that NSMase was involved in cell differentiation (Raines et al., 1993; Kim et al., 1991), activation (Mathias et al., 1993) and possibly proliferation (an implied, but not demonstrated *neutral* SMase effect)(Olivera et al., 1992), while ASMase caused apoptosis (Cifone et al., 1993). For example, Yang *et al* showed NFkB translocation occurred independently from PLC activation (Yang et al., 1993), which would exclude ASMase as an inducer of NFkB if PC-PLC (Machleidt et al., 1994) was the only means by which ASMase could be activated. Activation of SMases through different receptors e.g. TNFR (Wiegmann et al., 1994; Mathias et al., 1991), IL1R (Mathias et al., 1993) and Fas (CD95) (Cifone et al., 1993) may have their disparate functions controlled by the context of other signals generated by the receptor and also from subsequent metabolites of ceramide. For example, receptor ligation is often coupled to activation of PLC where its product DAG may activate PKC (Vance and Vance, 1991). PKC has been shown to protect endothelial cells against radiation induced apoptosis, most likely by inhibiting the apoptotic pathway rather than by enhancing DNA repair (Haimovitz-Friedman et al., 1994). In human myeloid leukaemia cells, it was found that activation of PKC (by DAG analogues or phorbol ester) arrested ceramide induced apoptosis at an early stage in the apoptotic pathway (Jarvis et al., 1994). The effects of ceramide and sphingoid bases (ceramide being an acylated version of sphingosine) on cell signalling may be more pervasive than currently accepted. Very high concentrations of sphingolipids, eg 330μM, *in vitro* inhibited or stimulated c-src, respectively, depending on the absence or presence of an acyl group (Igarashi et al., 1989) i.e. while sphingosine would inhibit src, ceramide would stimulate it. Until more precise experiments dissecting concentrations in different cellular compartments are performed, it may be equally justifiable to suggest high concentrations are unphysiological or that local concentrations of second messengers may reach a critical concentration to activate a src PTK or an alternative effect.

1.52 Ceramide and CD28

The wide acceptance that CD28 may be the key costimulus for T cell proliferation and activation has been thrown into some confusion by its role in a TCR-independent activation of ASMase (Boucher et al., 1995) because of the conflicting cellular responses arising from ceramide. ASMase activity has been linked to apoptosis (Cifone et al., 1993) but CD28 functions to costimulate activation and proliferation. Interestingly support for ASMase as a mediator of apoptosis may be provided by observations of lymphoblasts from Niemann-Pick patients. This disease arises from a deficiency of ASMase and results in the resistance of lymphoblasts to apoptosis following ionizing radiation (Santana et al., 1996), thereby implying ASMase is required to facilitate stress related apoptosis. Paradoxically many of the targets of ceramide, the product of sphingomyelin hydrolysis by ASMase, are shared by CD28. Ceramide is a potent sphingolipid second messenger with many proposed functions such as activation of PKCζ (Pushkareva et al., 1995) which leads to catabolism of IκBα. CD28 may be involved in PKCζ activation as it can activate PI3K (Stein et al., 1994). Activated PI3K can generate $PI_{3,4,5}P3$ which has been shown to activate PKCζ (Nakanishi et al., 1993). PKCζ may phosphorylate IκBα (Lozano et al., 1994) facilitating the subsequent translocation of NFκB to the nucleus (Boucher et al., 1995). NFkB nuclear translocation has been demonstrated to be both a CD28 derived event (Boucher et al., 1995) and may also be induced by ASMase (Boucher et al., 1995). A further role for ASMase in contributing to T cell activation is provided by the serine/ threonine kinase CAPK (ceramide activated protein kinase) which is activated by ceramide (Liu et al., 1994). CAPK has a preference for the minimal amino acid sequence -TLP such as found on EGFR and Raf. Raf was phosphorylated and activated by CAPK, suggesting ceramide may influence signals of the MAPK cascade (Liu et al., 1994)which are believed to contribute to positive regulation of T cell function. Another ceramide substrate is the ceramide activated protein phosphatase, CAPP; a class 2A PTPase which may be involved in anergy induction (Kolesnick and Fuks, 1995). CAPP and CAPK have been linked to anergy and apoptosis respectively (Kolesnick and Fuks, 1995; Pushkareva et al., 1995) while past data would indicate signals generated from CD28, leading to nuclear translocation of cytoplasmic elements of NFκB (Lai and Tan, 1994; Boucher et al.,

1995) are involved in cellular activation and proliferation rather than anergy or death. Data are not available on the effect of CD28 ligation on CAPP and CAPK. However it would be concordant if CD28 did not activate them due to their proposed involvement in apoptotic/ anergic pathways. Alternatively other signals may impinge on CD28-SMase derived effects. One possibility concerns the involvement of DAG-induced PKC activation (see section 1.51) which is proposed to rescue cells from apoptosis (Pushkareva et al., 1995; Kolesnick and Fuks, 1995), although no explanation as to the mode of action is offered. Clearly it cannot involve activation of the CD28-sensitive PKCζ as this is DAG insensitive (Pushkareva et al., 1995). Another possibility may be due to the existence of a number of forms of ceramide, which may be stimuli sensitive in their induction.

In T cell blasts ceramide appeared to share some of the costimulatory effects of CD28 (Boucher et al., 1995) while the proliferation of murine splenocytes after mitogenic stimulation in the presence of ceramide was suppressed (Felding-Habermann et al., 1990). However as IL2 secretion, IL2R expression and the affinity of IL2R for IL2 was unchanged it appeared that ceramide was specifically inhibiting cellular proliferation rather than activation. The differences in effect between ASMase on blasts and ceramide on splenocytes may be accounted for by different signals impinging on the intracellular environment. In the case of the splenocytes some of these may come for exogenous soluble factors or ligand-receptor interactions from neighbouring cells. The result of these influences may determine a pro- or anti-proliferative effect (fig. 1.9). Possible routes by which ceramide may exert an anti-proliferative effect are manifold. It may be converted to dimethylsphingosine (DMS) which strongly inhibits cell growth (Felding-Habermann et al., 1990). This would require deacylation of ceramide by ceramidase to generate sphingosine. A subsequent methylation of sphingosine would generate DMS (Vance and Vance, 1991). Alternatively by conversion to sphingosine, ceramide may inhibit PKC activity in resting and confluent cells, although not in rapidly dividing cells (Zhang et al., 1990).

In extraneural cells ceramide may be converted to glucosylceramide, followed by the addition of a large variety of sugar residues to form complex glycosphingolipids e.g. sialyl-glucosyl ceramide (G_{M3}) (Felding-Habermann et al., 1990; Hoffman-Ostenhoff et al., 1978). G_{M3} inhibits signal transduction by preventing EGF receptor dimerization and thus there is no activation of receptor-associated tyrosine kinase activity nor any activation of distal signals connected to mitogenesis (Hakomori, 1990).

A proliferative effect from ceramide may be facilitated by its conversion to sphingosine and subsequently by sphingosine kinase to sphingosine-1-phosphate (SPP) (Ghosh et al., 1990). SPP stimulated phospholipase D (PLD) which generated the potent mitogen phosphatidic acid (PA) from phosphatidylcholine (Fukami and Takenawa, 1992; Desai et al., 1992). Interestingly, in the presence of PMA, reduced levels of dihydrosphingosine (DHS, a sphingosine precursor) have been noted in human neutrophils with concomitant increases in ceramide levels (Wilson et al., 1988). The balance between generation and utilization of ceramide may be dependent on cell type and stage of activation. An alternative method by which proliferative signals may be prolonged may be invoked through PA. A cytoplasmic GTPase-activating protein (rasGAP), capable of converting active rasGTP to inactive rasGDP may be inhibited by PA provided PA has unsaturated fatty acid moieties such as arachidonic acid, which contains a 20 carbon chain with 4 *cis* double bonds (Tsai et al., 1989). Therefore ceramide may indirectly, through conversion to SPP, increase PA synthesis and prolong Ras activity. This presents an opportunity for modulation of PI3K activity as PI3K has been shown to be activated by Ras (Rodriguez-Viciana et al., 1994). Alternatively sphingolipids may alter T cell signalling due to the indirect activation of DAG kinase (DAGK) by sphingosine (Kanoh et al., 1990). Human PBMC and Jurkat T cell DAGK is predominantly located in the cytosol, although PMA can induce translocation of DAGK to the membrane where DAG is produced. As PMA can both activate PKC (Vance and Vance, 1991) and induce recruitment of DAGK, which decreases the levels of DAG following activation of DAGK by sphingosine (Kanoh et al., 1990), PKC activation may be indirectly controlled by sphingolipids. Therefore sphingolipids may exert a modulatory effect on elements of both the primary and costimulatory pathways involved in T cell activation.

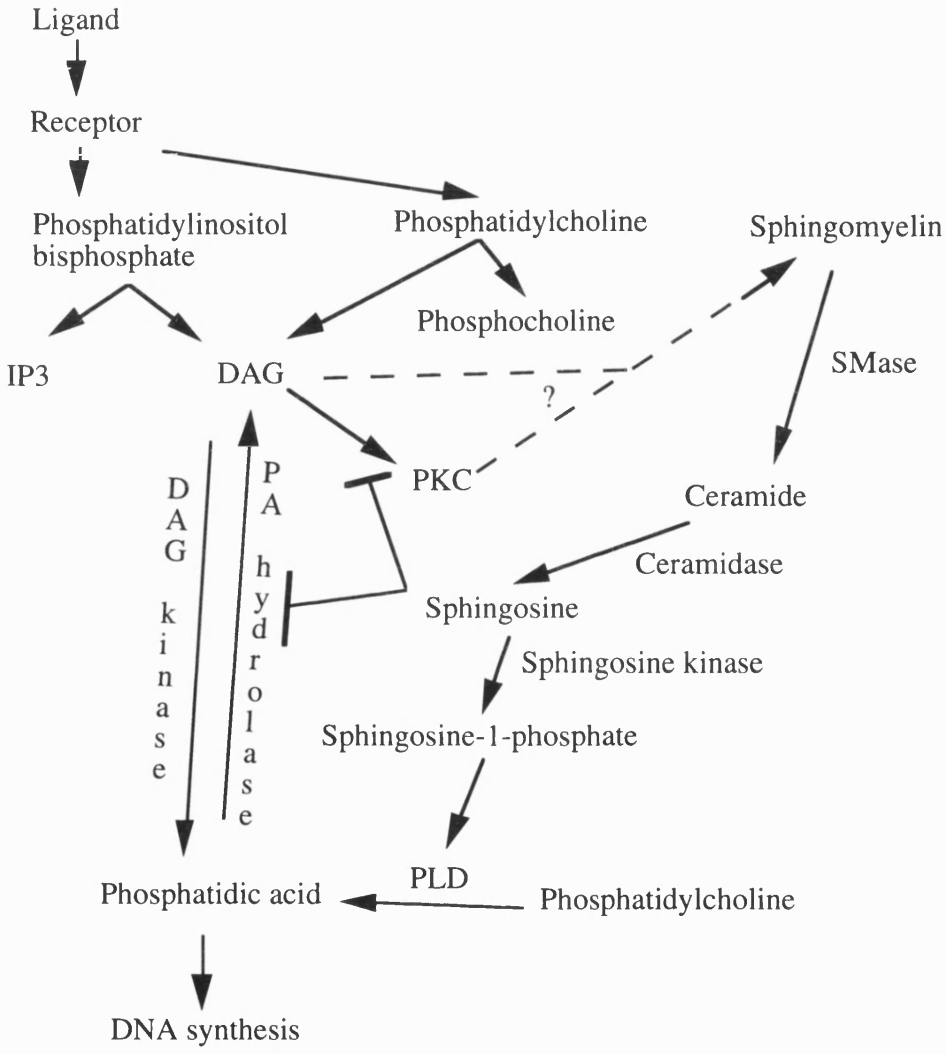


Fig. 1.9: Interrelationship of Phospholipid and Sphingolipid Signalling Pathways

Hydrolysis of membrane associated phospho- and sphingolipids generate signals which modulate each other through complex regulatory mechanisms

Sphingolipid metabolism may be seen to be exceedingly complex with paradoxical effects between different cell types e.g. ceramide, respectively, had negative and positive effects on T cell and fibroblast growth (Felding-Habermann et al., 1990; Olivera et al., 1992). Assay conditions may also give rise to apparently contradictory results e.g. exogenously added SMase induced NFkB translocation through ASMase but not NSMase while lysate preparations showed NFkB was induced through both N- and ASMase (Wiegmann et al., 1994; Machleidt et al., 1994). The acylation of ceramide to glycosphingolipids, or its transacylation (exchange of one fatty acid for another) or deacylation to sphingoid bases (Vance and Vance, 1991) presents many possible mechanisms by which ceramide may influence cell biology. The suggested negative regulatory function of CAPK (Mathias et al., 1991; Liu et al., 1994) and CAPP (Wolff et al., 1994) concurs with the activation of SMase by the receptors TNFR (Wiegmann et al., 1994; Kim et al., 1991; Yang et al., 1993) and Fas (Cifone et al., 1993) which have a negative role on cell viability. However the reported putative CD28-ASMase association (Boucher et al., 1995) would appear to be paradoxical due to the costimulatory role of CD28. So far, it is apparent that the effects of ceramide are context and cell type specific (Cifone et al., 1993; Yang et al., 1993; Igarashi et al., 1989) and little is known about the effects of ceramide in resting T cells.

1.6 Aims of the Project

The activation of T cells is highly regulated by processes, the identity and function of which are just emerging. Proximal signals generated from CD28 determine whether CD28 transduces anti-apoptotic, proliferative or activation messages to the T cell. Clearly T cells have stage specific sensitivity to costimulation and in the case of CD28, the most profound effects are seen on resting memory T cells where there is an absolute requirement for CD28 costimulation in order that high amounts of IL2 are produced from those T cells. Antigen-primed T cells however, also respond to CD28 derived signals, as is evident from their high levels of proliferation. In both these cases CD28 has effects beyond that seen by other costimulatory receptors such as CD2 or LFA-1.

The mechanism(s) by which CD28 transduces costimulatory signals are unclear, although part of the functional outcome of CD28 ligation may lie in promoting progression of cell cycling. Many mitogenic receptors functionally couple to PI3K while SMase also appears to be a candidate modulator of the growth of many cell types. For these reasons, it was decided to examine the effects of proximal signals associated with CD28 and sphingolipids which may affect T cell growth. The following parameters were analysed:

i) phosphorylation states of CD28 and associated molecules following CD28 ligation

ii) identity of molecules associating with CD28

iii) possible protein tyrosine kinase(s) facilitating the recruitment of proximal signalling elements to CD28

iiii) relationship between CD28 and sphingomyelinase

v) relationship between resting T cell costimulation and sphingomyelinase

vi) possible interplay of CD28 and sphingomyelinase on distal targets of both

It was hoped that data arising from such experiments would provide clues to the identity of the effectors and mechanisms by which CD28 transduced signals lead to the activation and proliferation of resting T cells.

Chapter 2

Methods

2.1 Reagents

Unless otherwise specified all laboratory reagents were purchased from Sigma (Poole, UK). Cell lines used included Jurkats, Chinese hamster ovary (CHO) K1, CD28 and CD80 expressing CHO transfectants as previously reported (Sansom et al., 1993). The antibodies used in the experiments were obtained from a variety of sources. OKT3 (αCD3), HB8784 (αCD25) and L243 (αHLA-DR) were obtained from ATCC (Rockville, MD, USA). UCHM1 (αCD14) was received from Prof. P Beverley (ICRF, London, UK); 9.3 (αCD28) and BB-1 (αB7-1) from Dr P Linsley and BU12 (αCD19) from Dr I. McLennan (University of Birmingham, UK). Monoclonal αCD69 (Cat. No. MCA1442) was purchased from Serotec, Oxford, UK. Western blotting primary polyclonal antibodies against fyn (Cat. No. sc-16; Santa Cruz; Autogen Bioclear UK Ltd, Wiltshire, UK), lck (donated by Dr C. Bebbington, Celltech, Slough, UK) and ZAP70 (Cat. No. ZU06-271; TCS Biologicals, Bucks, UK) were acquired from various sources and JS14 (αp85α) and 4G10 (α-phosphotyrosine) were donated by Dr M. Welham (University of Bath, UK). Secondary Western blotting goat α-mouse (P0447) / α-rabbit (P0448) antibodies were purchased from Dako (Glostrup, Denmark). 15E8 (αCD28) was donated by Dr van Liev (Amsterdam, Netherlands) and the GST-c-Jun fusion PGEX construct was supplied by J. Woodget, Toronto. Restriction enzymes were purchased from Promega Ltd, Southampton, UK and λ Hind III cut with EcoRI (Cat. No. 15612-013) markers to allow sizing of DNA fragments were purchased from Gibco BRL, Paisley, UK. λ Hind III fragment sizes are 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bps. For following the migration of proteins under SDS-PAGE, prestained markers were used (Cat. No. 160-0305; Biorad Laboratories, Herts, UK). Enhanced chemiluminescent (ECL) reagents (Cat. No. RPN2106) and ^{14}C sphingomyelin (Cat. No. CFA566) were purchased from Amersham (Amersham International, Bucks, UK). ^{32}P γ-ATP was purchased from ICN (4500 Ci/ mmol, Cat. No. 38101X, ICN, California, USA). Nitrocellulose membrane used in Western blotting was purchased from Anderman and Co. (Cat. No. 401236, Schleicher and Schuell; Anderman and Co., Surrey, UK). The following reagents were purchased from the suppliers indicated. PGNaseF (Cat. No. 704S; New England BioLabs, Herts, UK).

Protein A Sepharose beads (Cat. No. 17-0974-01, Pharmacia Biotech, Herts, UK). Biotin and avidin D.HRP (Cat. No. SP01210; Vector Laboratories, Peterborough, U.K). Rabbit complement (Cat.No. MA CL3221; Cedarlane Laboratories, Newcastle upon Tyne). Heparin (5000U/ ml, Monoparin CP Pharmaceuticals). Lymphoprep (density 1.077 g/ ml; Nycomed UK Ltd, Birmingham, UK).

2.2 Generating Protein Tyrosine Kinase Transfectants

Plasmid preparation

Plasmids based on pF10 containing fyn, lck or ZAP70 cloned downstream of human cytomegalovirus promoter were received from Dr C. Bebbington, Celltech, Slough (appendix 1). Additionally these plasmids contained a xanthine-guanine phospho-ribosyltransferase (gpt) gene facilitating selection of cells transfected with these plasmids under the regulation of XMAT (appendix 3) provided guanosine was omitted from the media (Mulligan and Berg, 1981).

Spectrophotometric determination (at OD_{260}nm) of the concentration of the plasmid preparations revealed that fyn and lck plasmids were at an insufficient concentration to proceed with transfection of mammalian cells. In order to generate more fyn and lck vector electrocompetent E. coli Top 10F cells were transformed by electroporation with either fyn or lck plasmid. To a 2mm width electroporation cuvette (Biorad, Herts, UK) sitting in ice, a 40 µl aliquot of Top 10F cells was added with 10-20ng of plasmid and mixed gently. At a resistance of 200Ω and a capacitance of 25µF, a potential difference of 2.5kV was discharged achieving a time constant of 3.5 ms. 950µl of SOC (appendix 6) was added to each cuvette and the cells were left at 37°C for 30 mins. Aliquots from 50 -100µl were plated out on ampicillin-containing agar plates (appendix 6) and left at 37°C overnight.

Several bacterial colonies were picked at random from the agar plates and grown overnight in 5ml broth minicultures (appendix 6). Minipreps were prepared from each using standard alkaline lysis protocols (Sambrook et al., 1989). Single restriction enzyme digests were carried out and analyzed on a 1x TAE buffered 1% agarose-ethidium bromide gel (appendices 4 and 5) to identify cultures carrying fyn or lck insert. A 500 ml maxiprep was prepared from one positive culture of each using the above method and the plasmid preparation was PEG purified (Sambrook et al., 1989). The plasmid pellet was resuspended in MilliQ H_2O and the presence and orientation of an insert was verified by single restriction enzyme digests. The products of which were

separated and visualized by an agarose-ethidium bromide gel. The concentration of the plasmids were measured by spectrophotometry at 260nm.

Transfection

CHO cells transfected with CD28 human cDNA as previously reported (Sansom et al., 1993) were transfected with fyn, lck or ZAP70 expression vectors. 3 million $CD28^+$CHO cells were washed in Hepes Buffered Saline (HBS, appendix 2) and resuspended in 5 mls of HBS. To a 4mm width electroporation cuvette (Biorad, Herts, UK), a 175 µl aliquot of $CD28^+$CHO cells was added with 5µg of plasmid and mixed gently. At a capacitance of 125µF, a potential difference of 350V was discharged, achieving a time constant of 3.5 ms. 1ml of glutamine free media (appendix 3) was added to each cuvette and the cells were left at room temperature for 4 mins. The cells were then transferred to cell culture flasks and selection was applied after one passage.

2.3 Cell Culture

Assays were carried out using the leukaemic T cell line Jurkat, purified resting T cells, CHO, $CD28^+$CHO, $CD80^+$CHO and PTK^+CD28^+CHO transfectants. Jurkats and T cells were cultured at 1-2x10^6 cells/ ml in RPMI 1640 containing 10% FCS supplemented with penicillin and streptomycin (appendix 3).

CHO cells, CD28 and CD80 transfectant cells as previously reported (Sansom et al., 1993) were cultured in glutamine free DMEM (Glu-) containing 10% FCS supplemented with nucleosides and sodium pyruvate (appendix 3). PTK transfectants were grown in Glu- media containing 10% FCS, sodium pyruvate, XMAT and nucleosides where guanosine was replaced with uridine (appendix 3). Selection of transfected cells in XMAT is based on the inhibition of purine synthesis by mycophenolic acid and the conversion of xanthine to XMP by the transfected gene product guanine phosphoribosyltransferase (Mulligan and Berg, 1981) which provides the cells with an alternative mechanism to generate GMP. Cells growing in XMAT were monitored regularly using FACS analysis of CD28 surface staining and Western blotting for PTK detection. The cells were passaged every 3 days by aspiration of the media followed by a

PBS wash and a 5 min incubation with trypsin at 37°C. The cells were dislodged by tapping the flask and media was added to inactivate the trypsin. Up to 90% of the cells were removed and the remainder diluted to volume with fresh media.

Cell lines and clones were maintained as stock by cryogenic storage. Following harvesting of subconfluent cells, they were washed in medium and resuspended in medium containing 20% FCS and 10% DMSO. Aliquots at $1x10^6$ cells/ ml were dispensed to freezing vials (Nunc) and transferred to storage at -80°C for 24 hours before indefinite storage in liquid nitrogen at -190°C.

Cell recovery from frozen was performed by rapid thawing under warm water and washing of the cells in medium, followed by gentle pelleting of the cells at 800 rpm for 5 mins. The cells were resuspended in 10mls of media, prewarmed to 37°C, before being introduced to a culture flask and the volume of media was adjusted to the appropriate volume for culture.

2.4 Flow Cytometric Analysis

Flow cytometric analysis (FACS) was used to provide a relative quantitation of constitutive and inducible antigen expression levels at the cell surface, thereby giving an indication of how stimuli may affect a cell or why a cell may respond in a particular way to a certain treatment. The analysis requires labelling of the surface antigen using specific mAbs followed by detection using a fluorescent labelled antibody which binds the primary antibody. When a sample is analyzed, the cells are directed through a LASER light source in a single cell suspension. Depending on the physical properties of the cell and the amount of antibody bound to the cell, the light is defracted to varying proportions. Relative to the plane of the LASER, information may be collected on how the light source is altered by forward scatter (FSC, related to cell size), side scatter (SSC, related to cellular granularity) and fluorescence from the labelled antibody (FL1, in this study used to assess surface antigen fluorescence levels and FL2, in this study used to measure propidium iodide entry in to a cell). A Becton Dickinson FACStar was used to record data using a 60mW LASER at 520nm for FL1 and 580nm for FL2.

Typically $2x10^5$ cells were stained for relevant markers while cells in medium were used as a negative control. Supernatants from hybridoma cell lines contained antibody concentrations of approximately 10 μg/ ml. Cells harvested from culture were washed in an excess of PBS, pelleted at 1500rpm for 5 mins (Beckman GPR) and resuspended in 50μl of primary antibody at 10μg/ ml. The cells were incubated at 4°C for 45 mins. Excess and unbound antibody was removed by washing the cells in PBS. The cells were pelleted and (as above) and the primary antibody labelled cells were secondarily labelled with 50μl of 10μg/ ml polyvalent α-mouse immunoglobulin conjugated fluorescein isothiocyanate (FITC) in the same manner as the primary antibody. Subsequently the cells were recovered and resuspended in 200μl of PBS before analysis. Normally 10000 events were recorded as a representative sample. Data are presented as histograms with the percentage expression above negative controls indicated in brackets. The percentage represents the level of fluorescence greater than the fluorescence recorded on the 95th percentile of the control treatment.

Homogeneous expression levels of CD28 on PTK transfectants would facilitate the analysis of the effect of one PTK compared to another. PTK transfectants following expansion under XMAT selection were assessed by FACS for levels of CD28 surface expression. Twenty clones of each type of PTK transfectant with comparable CD28 expression were directed into a 96 well flat bottomed plate at 1 cell/ well using a Becton Dickinson automatic cell deposition unit (ACDU) programme. The clones were expanded under XMAT selection where initially medium was added at 50μl/ well before transfer to 75 cm^2 culture flasks for further expansion.

2.5 Generating $CD28^{LOW}$ Jurkats

Antibody labelling of CD28 on the cell surface of Jurkats provided a useful mechanism by which $CD28^+$ve cells could be removed from a Jurkat population. $CD28^+$ve Jurkats labelled with the αCD28 mAb 9.3 were removed from the population by three methods. Firstly complement mediated lysis, secondly immunomagnetic depletion and thirdly by FACS sorting to exclude $CD28^+$ve cells. The treatments were performed sequentially

such that following the recovery in cell number of a treated population, the population was further treated to remove residual $CD28^+$ve Jurkats.

Complement mediated lysis of $CD28^+$ve Jurkats was initially used to decrease $CD28^+$ve cells from Jurkats. $5x10^6$ cells were labelled in 500µl of 9.3 (1/ 2000 dilution from ascites) for 40 mins at 4°C and subsequently washed in PBS. The cells were resuspended in 250µl of serum free medium and dispensed at 50µl/ well to a 96 well flat bottomed Petri dish. 1ml of ice cold water was added to lyophilized rabbit complement and the solubilized complement was filter sterilized. 50µl/ well of complement was added to the αCD28 labelled cells. The cells were incubated for 1 hour at 37°C before addition of 100µl/ well of R10 medium (appendix 3). The cells were left overnight at 37°C before expansion of the population for use in further CD28 depletion experiments. Following recovery of cell numbers, CD28 surface expression was assessed by FACS analysis. The cells were labelled Jurkat complement-treated cells Round 1-4, JcR1-4, depending upon how many rounds of complement treatment they had been subjected to. The complement mediated lysis of $CD28^+$ve cells was performed four times before an alternative method was used to further decrease $CD28^+$ve cells from the population.

Immunomagnetic depletion of $CD28^+$ve Jurkats was subsequently used. $5x10^6$ cells from the final round of complement treated Jurkats were taken and labelled with murine αCD28 antibody and washed in PBS. The cells were resuspended in 250µl of medium and 25µl of immunomagnetic sheep anti-mouse IgG beads (Dynal UK Ltd, Bromborough, UK) and rotated at 4°C for 2 hours. The $CD28^+$ve cells were removed from the population by a magnet and the resulting population was resuspended in 10mls of medium and expanded before analysis by FACS for levels of CD28 surface expression on the treated population (labelled JMAG).

FACS sorting was used to further deplete the number of cells in a Jurkat population with detectable CD28 surface expression. $2x10^5$ cells from the population subjected to magnetic depletion were stained for FACS analysis. Cells with low or no CD28 fluorescence were selected with a Becton Dickinson FACStar Plus ACDU programme.

Cells incubated with the primary αCD28 mAb which had lower fluorescence than the negative control were directed into a 96 well flat bottomed flat bottomed microtitre plate and subsequently expanded. This method of selecting CD28 -ve/ low expressing Jurkats was repeated and the final population, labelled CD28 Negative Jurkats, 28N, was cryopreserved following FACS analysis of surface phenotype compared to the parental J16 population.

2.6 Western Blotting

Western blotting may be used to detect the presence of proteins to which an antibody has been developed. Protein detection is based upon the use of a primary antibody directed against an antigen which is detected by a secondary antibody targeted against the primary antibody. The relative level of protein is visualized in a reaction where the secondary antibody conjugated with a horse radish peroxidase (HRP) activity generates free radicals upon incubation with substrate e.g. hydrogen peroxide. The reaction products can expose X ray film. Proteins from cell lysates or immunoprecipitates which are separated by electrophoresis and transferred to a nitrocellulose membrane may be incubated with Western blotting antibodies and detection reagents in order to generate a record of the level of protein detected upon exposure of X ray film to the activated detection reagents.

Sample Preparation

For the detection of fyn, lck, ZAP70 and p85 proteins from cell lysates, 1 million cells were washed twice in serum free medium and lysed in 30μl of lysis buffer (appendix 4). The samples were incubated on ice for 40 mins before pelleting cytoskeletal and nuclear proteins at 14000rpm, 4°C for 6 mins (Beckman G15R). The post-nuclear supernatant (PNS) was mixed with an equal volume of loading buffer and boiled at 100°C for 10 mins. The samples and 10μl of prestained markers were loaded on a 1mm thick SDS-PAGE gel comprising a 4% acrylamide stacking gel (to a depth of 1cm) and a 12% resolving gel. Samples were electrophoresed at 10mA through the stacking gel and 20mA through the resolving gel under constant current in running buffer (appendix 4).

Detection of tyrosine phosphorylation changes in OKT3-stimulated Jurkats was assayed in a similar manner, except before lysis the cells were incubated at 37°C from 0-30 mins with the αCD3 mAb OKT3 or an irrelevant antibody L243 (αHLA-DR) at a final concentration of 10μg/ ml. The stimulations were carried out in 10μl of serum free RPMI and stopped by the addition of an equal volume of lysis buffer. The samples were precleared on ice and the PNS was mixed with 20μl of loading buffer, boiled and separated on a 1mm thick 4% stacking/ 12% resolving gel.

Tranfer

Following electrophoresis the separated proteins were transferred from the gel to a nitrocellulose membrane. The proteins carry a net negative charge and so when a current is applied across them, they migrate from the negative to the positive electrode. The migrating proteins may be transferred to a nitrocellulose membrane placed between the gel and the positive electrode. A cassette was used to support a 'sandwich' of a foam (Scotchbrite) pad, two sheets of Whatman blotting paper, the gel, the membrane, two sheets of Whatman and a foam pad. All elements of the sandwich were presoaked in transfer buffer (appendix 4) before assembly and the cassette submerged in transfer buffer with the gel oriented to the negative electrode and membrane toward the positive electrode. A cooling coil with self-contained circulating fluid at 4°C was placed in the transfer tank to limit a rise in temperature which may adversely affect the structure of transferred proteins. Transfer was performed at 0.6A for 1.5 hours.

Subsequently the efficiency of transfer was assessed by staining the gel for half an hour in Coomassie stain with three half hour washes in destaining solution (appendix 2). This was performed on a rocking platform at room temperature. The gel was dried at 70°C for 1 hour under vacuum on a gel drier (Biorad, Herts, UK)

Blotting

To prevent non-specific signals from arising, the membrane with the blocked by incubation in 100mls of blocking buffer comprising 5% w/v BSA and 0.01% w/v Na azide in TBS for 3 hours on a rocking platform before being washed in 100mls of TBS

for 10mins. Primary antibody was diluted in a 10% v/v blocking buffer solution in TBS and membranes were incubated for 2 hours in 5mls of diluted primary antibody (final dilutions indicated in the results) with rocking. After which the membrane was washed for 10 min/ wash once in TBS, 3 x TBS with 0.1% v/v NP40 (TBSN) and once again in TBS. All washes were with 100mls of TBS or TBSN. Secondary antibody was diluted in TBSN and the membranes was incubated in 10mls of this for a further 2 hours before washing as before. An additional TBS wash was performed as well and then the membrane was incubated with ECL reagents for 1 min. The membrane was drained of excess reagent and used to expose X ray film.

2.7 Immunoprecipitation of CD28

Precoupling αCD28 Antibody to Protein A Sepharose Beads

To isolate and enhance the detection of signalling molecules associated with CD28, CD28 was immunoprecipitated using an antibody (15E8) immobilized on Protein A Sepharose beads. The beads supplied as a lyophilized powder were rehydrated according to the manufacturer's instructions and an equal volume of 80% PBS and 20% ethanol v/v storage buffer was dispensed to them. 1ml of a 50% v/v slurry of Protein A beads was washed gently in PBS three times before 200µg of 15E8 was added to them and the volume adjusted to 1ml with PBS. The beads and antibody were left to bind by rotation at 4°C overnight. The first supernatant was retained and the beads were washed three times in PBS before resuspension in 0.5mls of storage buffer and stored at 4°C. The level of antibody binding to beads was found to be complete when antibody levels were assessed by Coomassie staining of SDS-PAGE separation of aliquots of the first supernatant and the precoupled beads.

Immunoprecipitation of Biotinylated CD28

Due to a paucity of antibodies suitable for detection of CD28 by Western blotting, an alternative method was used to determine the ability of the precoupled 15E8: Protein A beads to immunoprecipitate a protein matching the predicted molecular mass of CD28, which was 44 kDa (Aruffo and Seed, 1987). Biotin reacts with amino groups on

proteins and by immunoprecipitation of biotinylated protein, products may be visualized by SDS-PAGE separation and Western blotting. The blotting antibody, an avidin D and horse radish peroxidase (HRP) conjugate, served to bind biotin by an interaction between avidin D and biotinylated proteins and generate signals which exposed X ray film by an interaction between HRP and Amersham ECL reagents.

Typically $CD28^+$CHO cells or as a negative control non-transfected CHO cells at $5x10^6$ cells/ sample were biotinylated by washing in PBS.CM (PBS with 0.1mM $CaCl_2$ and 1mM $MgCl_2$), pelleted at 1500 rpm (Beckman) and resuspended in 0.5 ml PBS.CM. 20µl of a freshly made 50 mg/ ml solution of biotin was added to the cells, mixed gently by swirling and incubated at room temperature for 2 hours. Subsequently the cells were washed three times in PBS, resuspended in 1ml of lysis buffer (appendix 4), incubated at 0°C for 40 mins, before pelleting cytoskeletal and nuclear proteins at 14000 rpm for 2 mins. The supernatant was precleared of cross-reactive proteins which may have bound Protein A by a 4°C, 30 min rotation with 20µl of a 50% (vol/ vol) slurry of Protein A beads alone i.e. which had not bound antibody. The precleared supernatant was recovered and rotated overnight at 4°C with 15E8: Protein A beads. Subsequently the beads were washed four times in lysis buffer by centrifugation at 6500 rpm, 10 second pulses and the supernatant discarded. The beads were resuspended in an equal volume of loading buffer, boiled for 10 minutes and analyzed by Western blotting using avidin D. HRP at 5µg/ ml as the Western blotting antibody. Upon generation of a new batch of precoupled beads, a number of points (at $5x10^6$ cell equivalents) were incubated with a titration of precoupled 15E8: Protein A beads to optimize immunoprecipitation of the maximum level of CD28, as indicated in the results.

CD28 Immunoprecipitation Experiments

A number of experiments were performed by stimulation of CD28 using fixed $CD80^+$CHO cells where the ratio of stimulatory to stimulated cells was 1:3. $CD28^+$CHO cells, PTK transfected $CD28^+$CHO cells or non-transfected CHO cells (as a negative control) were stimulated by fixed $CD80^+$CHOs or fixed CHOs for 5 mins at 37°C. Subsequently the cells were lysed in 1ml of lysis buffer (appendix 4) and incubated over

ice for 40 mins, before pelleting nuclear and cytoskeletal proteins and preclearing the solubilized proteins with 20 µl of a 50% v/v slurry of protein A beads. CD28 was immunoprecipitated by rotation of the precleared supernatants for two hours with precoupled 15E8: Protein A beads. The beads were washed four times in solubilization buffer before further treatment.

In order to assess changes in tyrosine phosphorylation of CD28 and/ or associated proteins, $2.5x10^7$ cells were stimulated by $CD80^+$CHOs or CHOs. 100µl of precoupled 15E8: Protein A was used to immunoprecipitate CD28. The beads recovered after immunoprecipitation and washing were loaded in 50µl of loading buffer (appendix 4), before being boiled for 10 mins and analyzed by SDS-PAGE and Western blotting with an anti-phosphotyrosine primary antibody G418 at a 1/1600 dilution and secondary antibody goat α-mouse.HRP conjugate (Dako) at 1/ 20000 dilution.

Recruitment of p85 the regulatory subunit of PI3K was assessed in a similar manner to changes in tyrosine phosphorylation except $1x10^7$ cells were stimulated and 40µl of 15E8: Protein A beads were used to immunoprecipitate CD28 from the cell lysates. The primary antibody, JS14, directed against p85α of PI3K and was used at a dilution of 1/2000 and secondary antibody goat α-rabbit.HRP conjugate (Dako) at 1/ 20000 dilution.

In Vitro Kinase Assay

Changes occurring in tyrosine/ threonine/ serine phosphorylation were assessed by performing in vitro kinase (IVK) reactions with CD28 immunoprecipitates. Cells were stimulated and immunoprecipitated in a manner analogous to that performed in detection of p85 recruitment to CD28. However after washing the immunoprecipitates the cells were washed once in cold, i.e. without [^{32}P γ] ATP, kinase buffer (appendix 2), before resuspension in 30µl of kinase buffer containing 2µCi [^{32}P γ] ATP. The cells were incubated at room temperature for 30 mins. The reaction was stopped by washing the immunoprecipitates in kinase buffer containing 30mM EDTA. The samples were washed twice more in this before resuspension in 20µl of loading buffer and boiling of the

samples for 10 mins. Proteins were separated by SDS-PAGE and following drying of the gel, phosphoproteins were visualized by autoradiography.

Deglycosylation of CD28 Immunoprecipitates

Following autoradiography of the products of a CD28 IVK assay, a number of proteins of different molecular weights were seen to have undergone increased phosphorylation due to CD80 stimulation. One of which, migrated as a broad band between 44-54 kDa. To determine whether the breadth of this band was associated with the protein being glycosylated, CD28 immunoprecipitates were treated with/ without PGNase F, a deglycosylation reagent which is an amidase capable of cleaving mannose and complex oligosaccharides from N-linked glycoproteins. $CD28^{+}$CHOs ($1x10^{7}$) were stimulated as in previous IVK assays with/ without CD80, subjected to an IVK reaction, followed by deglycosylation of the reaction products by PGNaseF. Subsequent to termination of the IVK reaction of CD28 immunoprecipitates and the removal of unbound/ excess label by washing in kinase buffer the samples were washed twice in RIPA buffer:

RIPA buffer:
1% v/v Triton X100
0.5% w/v sodium deoxycholate
0.1% w/v SDS
150mM NaCl
50mM Tris.HCl pH8
1mM Na orthovanadate

and protease inhibitors
10 μg/ ml leupeptin
10 μg/ ml aprotinin
100 μg/ ml soybean trypsin inhibitor

once in RIPA with 1M NaCl, twice in RIPA and once in buffer D:

buffer D:
0.1% v/v Triton X100

150mM NaCl

50mM Tris.HCl pH8

1mM Na orthovanadate

and protease inhibitors as above

The immunoprecipitates were resuspended in 20µl buffer D and incubated for 10 mins at 100°C to denature them. 2.5µl of 10X G7 buffer (manufacturer supplied) and 2.5µl of 10% v/v NP40 were added to the denatured samples. Each sample was incubated with PGNaseF at 1000U for 1 hour at 37°C. PGNaseF was supplied at 500U/ µl where 1U is defined as the amount of enzyme required to remove >95% of the carbohydrate from 10µg of denatured RNase B in a 10µl reaction at 37°C in 1 hour. An equal volume of loading buffer (appendix 4) was added to each point which were then boiled for 10 mins at 100°C before analysis by SDS-PAGE and autoradiography.

2.8 Modulation of Costimulation by Sphingomyelinase/ C2 Ceramide

Preparation of Purified Resting T cells

50 mls of blood from healthy volunteers was collected in heparinized tubes (0.1% v/v of 5000U/ ml) and diluted with an equal volume of PBS. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by aliquoting in the following proportions, 25mls of the blood and PBS mixture onto 15mls of a lymphoprep gradient (density 1.077 g/ ml) and centrifuged without a brake for 30 mins at 1500 rpm (Mistral 2L). Serum (top layer) was aspirated and the buff layer collected. This layer which comprised PBMCs was washed three times in RPMI containing 10% FCS. The cells were counted using a Neubauer haemocytometer and added to plastic Petri dishes at a dilution of no more than $2.5x10^6$ cells/ ml in medium. The cells were incubated at 37°C for 1 hour to remove adherent cells such as monocytes. The non-adherent cells were carefully removed, pelleted and resuspended in 500µl each of mAbs against activated T cells (L243, HLA-DR at 10µg/ ml), monocytes (UCHM1, αCD14 at 10µg/ ml) and B

cells using 10µl of αCD19 (BU12, a 1/10 dilution from ascites). The cells were rotated for 1 hour at 4°C and then washed in PBS before resuspension in 500µl of medium. 50µl of immunomagnetic sheep anti-mouse IgG beads were added to the cells which were rotated for another hour at 4°C before addition of 10mls media and removal of antibody labelled cells using a magnet. The cells were washed, counted and analyzed by FACS. Typical preparations were >95% $CD3^+$ve.

Proliferation Assays

The effects of different stimuli on cell growth may be examined using a proliferation assay. A dividing cell synthesises a copy of its DNA before mitosis and so requires nucleotides to facilitate DNA replication. Thymidine is one of the nucleotides required in DNA synthesis and the level of ^3H thymidine incorporation into cellular DNA is proportional to the amount of proliferation occurring. Analysis of the effect of various reagents on T cell proliferation may be performed by supplying each point with ^3H thymidine and allowing time for variations in the uptake of thymidine between treatments to become manifest before harvesting the assay and assessing the relative levels of radioactivity associated with one treatment compared to another. Thus the effect of one treatment compared to another on proliferation can be assessed.

The use of $CD80^+$CHO cells to provide a costimulatory ligand requires fixing of the cells to prevent high background counts due to their proliferation. Fixing kills the cell preventing further metabolism but allows the presentation of surface markers to occur i.e. the interaction of CD80 on $CD80^+$CHOs with CD28 on T cells is not prevented by fixing the $CD80^+$CHOs. Cells were fixed by washing harvested transfectants twice in PBS and resuspension of $5x10^6$ cells in 1ml of 0.025% v/v glutaraldehyde (in PBS). The cells were incubated at room temperature for 2 mins with occasional swirling to ensure adequate resuspension of the cells. The cells were washed twice in medium to remove excess glutaraldehyde and resuspended at the required concentration for the assay. Control wells of fixed $CD80^+$CHOs with T cells were included in assays to ensure that the levels of thymidine incorporation recorded were not attributable to transfectant proliferation and that proliferation of T cells arose from cells of a resting phenotype.

Typically a proliferation assay was performed by the addition of $5x10^4$ resting T cells with $1.7x10^4$ fixed $CD80^+CHO$ and αCD3 mAb at 10μg/ ml in RPMI medium. To determine whether the proliferation response of T cells to SMase or C2 ceramide was dependent upon the type of primary stimulus, the assay was duplicated but αCD3 mAb was replaced by PMA at 40ng/ ml as the primary stimulus. Sample volumes were adjusted to 200μl with 1x RPMI and aliquoted to a well in a 96 well flat bottomed microtitre plate. Treatments were performed in triplicate and for the last 8 hours of culture 1μCi of ^3H thymidine (5 Ci/ mmol, Cat. No. 2406605, ICN) was added to each well. Following this the cells were harvested onto glass fibre filters, separated into tubes with 500μl of scintillation fluid (Wallac, Optiscint HiSafe) and the radioactivity was measured using a β liquid scintillation counter.

The effects of various reagents as indicated in the results, were assessed in proliferation assays. The concentrations listed in the results refer to the final concentration of reagent in each well. All samples were equalized for vehicle at a concentration equivalent to the highest dose of reagent under analysis.

Viability Assay

In order to determine the effect of a reagent on cell viability in the proliferation assays, cells were analyzed for their ability to exclude propidium iodide (PI) from entry in to the cell. Cells which loose membrane integrity cannot exclude PI and show increased fluorescence (FL2) due to excitation of PI at 580nm i.e. mortality is proportional to FL2.

Cell viability was assessed using replicate plates from proliferation assays, except that ^3H thymidine was omitted. The triplicate points from each treatment were combined and PI was added to a final concentration of 10μg/ ml. Each treatment was analyzed by FACS and the data were recorded as FL2. Typically 10000 events were recorded as a representative sample. In the case of experiments where fixed $CD80^+CHOs$ were included, to avoid high background FL2 from the dead fixed cells, these cells were excluded from viability analysis on the basis of their larger size (FSC). By excluding the

very large FSC data from the analysis, little background data due to $CD80^+$CHOs was recorded.

Surface Marker Changes

To analyze the effect of reagents upon T cell phenotype the surface expression of CD25 and CD69 were assessed. Experimental conditions comparable to those for the proliferation assays were used except coated αCD3 mAb was used as the primary stimulus where the effects of αCD3 stimulation were analyzed. Soluble antibody would have been inappropriate due to interference with subsequent FACS assessment of CD25 and CD69 expression levels.

Typically a 24 well flat bottomed culture dish was coated in 100µl of 10µg/ ml αCD3 mAb for 4 hours at 37°C (or overnight at 4°C). Excess antibody was removed and the wells were washed twice by the gentle pipetting of 1ml PBS in and out of the well. $1x10^6$ T cells and 3.3 $x10^5$ $CD80^+$CHOs/ well were dispensed in 1ml of RPMI. The effect of SMase/ C2 ceramide on surface marker expression were determined by addition to replicate cultures. Over 4 days the treatments examined were costimulated cells with medium, $7.5x10^{-5}$ U/ ml SMase, 30µM C2 ceramide and 0.3% v/v ceramide vehicle (ethanol). Daily one well/ treatment was harvested, taking care not to scrape the coated αCD3 mAb off the bottom of the well. The cells were washed in PBS and divided into 3 equal aliquots for FACS analysis. One point was the negative control i.e. not stained with a primary antibody and the remaining two points were stained with 50µl of primary antibodies against CD25 and CD69 at 10µg/ ml. All treatments were secondarily stained with 50µl of 10µg/ ml goat α-mouse polyvalent FITC conjugate. Fluorescence data were recorded as FL1 where 10000 events were recorded as a representative sample.

To assess whether the primary stimulus for T cell proliferation regulated the effects of SMase/ C2 ceramide on T cell phenotype, a replicate assay was performed where the αCD3 mAb stimulus was replaced with PMA. PMA was added directly to the wells in a soluble form at a final concentration of 40ng/ ml. All other conditions were comparable

to the assay where αCD3 mAb was the primary stimulus. FL1 data were collected on CD25 and CD69 surface expression.

2.9 Sphingomyelinase Assay

Sphingomyelinase has been reported to be activated by receptors with opposing function e.g. CD28 (Boucher et al., 1995; Chan and Ochi, 1995) and Fas (Cifone et al., 1993). Sphingomyelinase generates ceramide and phosphocholine by the hydrolysis of sphingomyelin, a ubiquitous animal membrane component (Vance and Vance, 1991). An assessment of the degree of SMase activation was performed based on the detection of phosphocholine levels in aqueous cell extracts (Wiegmann et al., 1994). Stimulated cell lysates, incubated with ^{14}C sphingomyelin, may have their radiolabelled products detected by scintillation.

$CD28^+$CHOs were incubated in serum free glutamine free medium at 37°C for 2 hours to prevent serum-induced enzyme activation. 5 million cells in 100µl aliquots of serum free medium were stimulated by $1.7x10^6$ fixed $CD80^+$CHOs or untransfected CHOs. At the relevant time points the reactions were stopped by immersion of the eppendorf in liquid nitrogen, addition of 1ml of ice cold PBS and centrifugation at 2000rpm, 4°C (Heraeus Biofuge Fresco) for 15 seconds. The cell pellet was washed twice in PBS and once in a buffer A:

Buffer A	10%v/v Nonidet P-40
	1µg/ml leupeptin
	1µg/ml pepstatin
	1mM PMSF

Following centrifugation as before, the pellet was resuspended in 200µl of the same buffer on ice for 15 mins. The samples were pelleted at 13000rpm for 5 mins and 37µl of the supernatant was saved. The supernatant was incubated for 2 hours at 37°C in ASMase reaction buffer:

ASMase reaction buffer, pH 4.5	250mM Na acetate, pH 4
	1mM EDTA, pH 5

containing 1.1μCi/ ml ^{14}C sphingomyelin in a final reaction volume of 50μl. Phosphocholine was extracted from the samples by chloroform: methanol extraction (2:1). 800μl of chloroform: methanol were added to each sample with 300μl of H_2O. The samples were vortexed and centrifuged at 13000rpm. 200μl of aqueous phase were added to vials to which 5mls of scintillation fluid were added before counting. A time course of $CD80^+$CHO-induced enzyme activation of ASMase in responding cells was plotted as percentage increase in activity above the levels obtained through stimulation in the absence of CD80 i.e. with non-transfected CHOs.

In assessment of the neutral (N) and acidic (A) SMase activity in S. aureus SMase a comparable protocol was followed. To assess ASMase activity, enzyme aliquots were diluted in 50μl ASMase reaction buffer, pH 4.5 and to assess NSMase activity enzyme aliquots were diluted in 50μl NSMase reaction buffer pH 7.4:

NSMase reaction buffer, pH 7.4	20mM Hepes pH 7.4
	10mM $MgCl_2$
	2mM EDTA
	5mM DTT
	0.1mM Na_3VO_4
	0.1mM Na_2MoO_4
	30mM p-nitrophenylphosphate
	10mM β-glycerophosphate
	750 μM ATP
	0.2% v/v Triton X-100
	protease inhibitors as for buffer A

containing 1.1μCi/ ml ^{14}C sphingomyelin in a final reaction volume of 50μl. Following a 2 hour incubation at 37°C, phosphocholine was recovered by chloroform: methanol

extraction. A dose response of NSMase and ASMase activity in S. aureus SMase was plotted and compared against a simultaneous experiment using boiled S. aureus SMase.

2.10 Modulation of JNK Activity by Sphingomyelinase/ C2 Ceramide

JNK activation can be detected as a phosphorylation of c-Jun, a substrate of the JNK family members (Verheij et al., 1996). Lysates from stimulated cells incubated with a fusion protein GST.c-Jun, comprising the amino acids 5-89 of the N terminus of c-Jun, may reveal a differential ability between reagents to modulate JNK activity. An in vitro kinase reaction utilizing glutathione agarose beads (GAB) to immobilize the substrate facilitates the detection of radioactive phosphate utilized by JNK family members to phosphorylate GST.c-Jun. The products may be visualized by SDS-PAGE and autoradiography.

Induction of GST.c-Jun

25 mls of liquid broth medium containing 50µg/ ml ampicillin (LBamp, appendix 6) was inoculated from a thawed stock of E.coli transfected with a vector containing an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter regulating the expression of GST.c-Jun. The inoculum was shaken overnight at 37°C. Two flasks of 400mls LBamp were inoculated with 12 mls each of the culture and grown for 2-3 hours until the OD_{600}nm reached 0.6 to 0.8 but not more than 0.9. 2mls of culture were removed for continued growth without induction. Expression of GST.c-Jun was induced by addition of 0.4mM IPTG and continued growth for 3-5 hours at 37°C. A shorter time induces less protein but the protein may be more soluble. 1ml of uninduced and induced culture were transferred to separate eppendorfs, pelleted, resuspended in 80µl of loading buffer (appendix 4) and boiled for 10 mins. The samples were resolved by SDS-PAGE and the gel Coomassie stained to determine the presence of an induced protein in the IPTG treated culture.

Each of the 400ml induced cultures were pelleted at 4°C, 4000rpm for 20mins and the pellet resuspended in 10ml lysis buffer by pipetting up and down.

Bacterial lysis buffer:	1%v/v Triton X-100
	2mM EDTA
	1mM PMSF
	1μg/ml leupeptin
	1μg/ml pepstatin

The lysates were combined and stored overnight at -20°C before sonication for 40 secs. The samples were sonicated three more times with a 1 min interval on ice between each sonication. The samples were pelleted at 10000rpm, 4°C for 10 mins and 2ml of a 50% slurry of presoaked GABs (in PBS) were added to the supernatant. The mixture was rotated for 2 hours at 4°C, before gentle pelleting of the beads for 15 secs at 1200 rpm. A 2 min interval was allowed for the beads to settle before aspirating the supernatant. The beads were washed once in lysis buffer (as above) by rotation at 4°C for 5 mins and washed (without rotation) again in lysis buffer, three times in PBS containing 2mM EDTA, three times in PBS and once in storage buffer.

GAB storage buffer:	50mM Hepes pH 7.4
	50mM NaCl
	50% v/v glycerol

The final wash in storage buffer was performed for 1 min at 1200rpm and an interval of at least 10 mins was allowed before careful aspiration of the supernatant. The beads were transferred to two eppendorfs at 500μl beads/ eppendorf and an equal volume of storage buffer was added to each eppendorf. The beads were stored at -20°C ready for use where 10μl of the 50% v/v bead slurry were used.

Measuring GST.c-Jun Phosphorylation

Initially the sensitivity of the newly made batch of GST.c-Jun beads to stimuli was assessed. Stimulation with/ without a combination of 5ng/ ml PMA and 1μM ionomycin (Cat. No. 407950, Calbiochem) was used to determine whether the old and new batches

of GST.c-Jun beads could detect JNK activation. Furthermore as ceramide had been reported to stimulate JNK activation (Verheij et al., 1996), a dose response of C2 ceramide on the new beads was made. To account for a vehicle effect on JNK activity, the concentration of the vehicle was standardized at 0.5% v/v ethanol in all C2 ceramide treated samples. $1x10^7$ Jurkats were stimulated for 20 mins at 37°C before being pelleted at 1500rpm and 4°C, washed in PBS, repelleted and lysed in 1ml of lysis buffer (appendix 4). Lysates were stored on ice for 40 mins before pelleting nuclear and cytoskeletal protein at 14000rpm for 6 mins at 4°C. The supernatants were rotated with 10µl of GST.c-Jun beads for 2 hours at 4°C. The beads were washed three times in lysis buffer, leaving 1 min after each centrifugation to allow the beads to settle before aspirating the supernatant, and once in JNK kinase buffer (appendix 2). The beads were resuspended in 35µl of kinase buffer containing 2µCi of ^{32}P γ-ATP and incubated at 37°C for 30 mins. The reaction was stopped by the addition of 40µl of loading buffer and boiling for 10 mins. The samples were separated by SDS-PAGE, the gel dried and the degree of c-Jun phosphorylation visualized by autoradiography. Subsequently the gel was rehydrated by immersion in 750mM Tris.HCl pH 8.8 for 10 mins before being Coomassie stained to determine protein loading.

After validating the new batch of beads JNK activity was assessed in resting T cells under a variety of stimuli. $1x10^7$ resting T cells were stimulated for 20 mins at 37°C by combinations of PMA (40ng/ ml), αCD3 mAb (diluted 1/100 from ascites), $3.3x10^6$ fixed $CD80^+$CHOs, $7.5x10^{-5}$ U/ ml SMase, 30µM C2 ceramide or 0.3% v/v ethanol. Following stimulation the resting T cell samples were lyzed and treated as for analysis of JNK activity in Jurkats.

Chapter 3

Generation of Cell Lines to Assess CD28 Signals

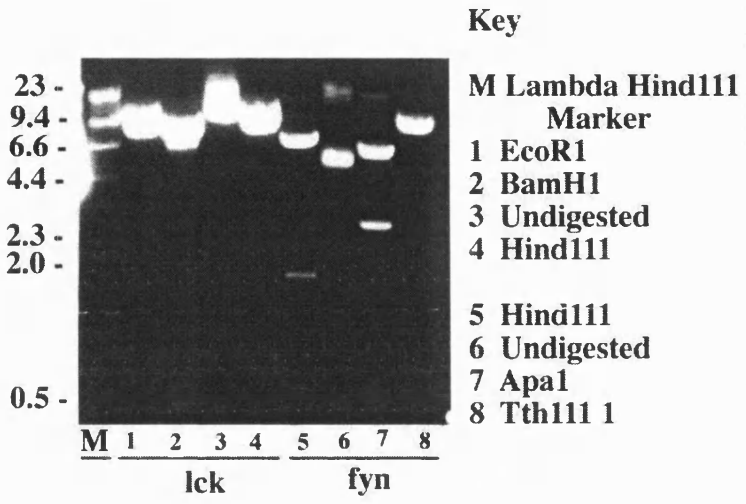
CD28 has been demonstrated to be reliant on tyrosine phosphorylation to transduce its costimulatory effect on IL2 secretion (Vandenberghe et al., 1992). It may be possible that the TCR and CD28 share the same PTKs in transducing their proximal signals, as neither receptor has intrinsic kinase activity. An analysis of if and what PTKs were involved in CD28 signalling was made by constructing a T cell model. A CHO cell line was chosen which had previously been transfected with, and expressed, CD28 (Sansom et al., 1993). The fibroblastic CHO cell line was used because it is not of haematopoietic origin and therefore CHO transfectants would be unlikely to have elements which would substitute for the function of one or other of the TCR-activated PTKs. Therefore the contribution of individual PTKs to CD28 signalling could be assessed. Thus a $CD28^{+}$CHO cell line was used as the parental cell line for a number of transfections with vectors expressing fyn, lck or ZAP70 cDNA in order to determine what contribution each of these PTKs might make to CD28 signalling.

Another approach adopted to identify how CD28 might transduce its costimulatory function was through the generation of a CD28 negative T cell line. It was hoped that this would be the basis for a T cell model with many of the functions and responses of a resting T cell remaining but without the CD28-initiated signals. This model would be useful in assessing which regions of the intracellular domain of CD28 were involved in recruitment/ activation of various effectors to CD28 subsequent to transfections with CD28 cytoplasmic domain mutants.

3.1 Plasmid Maxipreparation and Orientation of Fyn and Lck Vectors

In order to transfect the parental $CD28^{+}$CHO cell line with PTKs, a number of plasmids which expressed fyn, lck or ZAP70 were prepared for transfection. Maxipreparations of lck and fyn plasmid DNA were performed from E.coli transfected with the vectors. The DNA preparations were analyzed by restriction digestions to confirm whether or not there were i) fragments of a size compatible with a fyn or lck cDNA insert in the vector and ii) whether the insert was correctly oriented. Figure 3.1a shows the result obtained for each plasmid after various restriction enzyme digests were performed and the products separated by agarose gel electrophoresis.

a



b

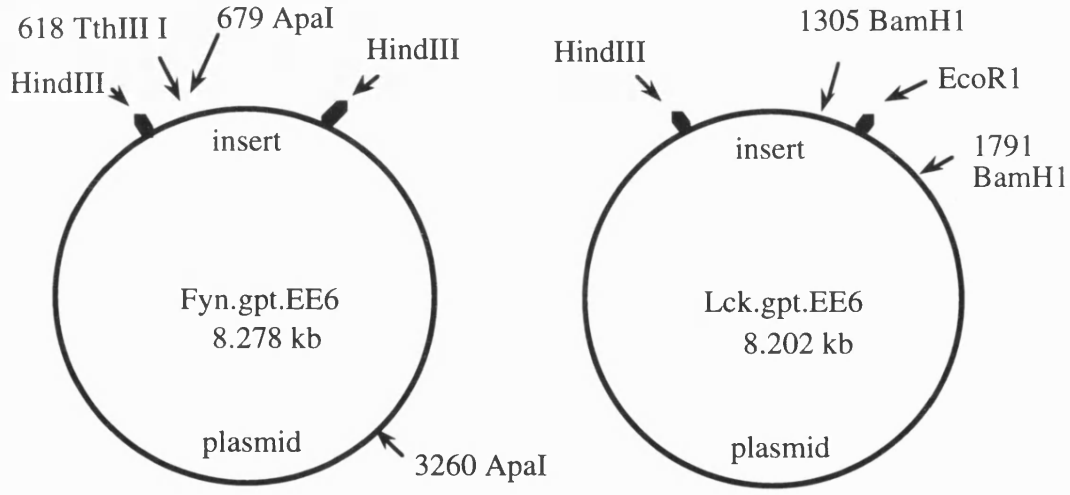


Fig. 3.1: Insert and Orientation Digests and Maps of Lck and Fyn Vectors

a) 1µg of lck and fyn vectors were digested by restriction enzymes (REs) as indicated and the reaction products were separated by TAE ethidium bromide agarose gel electrophoresis using 1µg of EcoRI cut λHindIII DNA marker to determine product size. b) A representation of the RE sites on each of the vectors with inserts of fyn (1.626 kb) and lck (1.55 kb) cDNA in sense orientation within a gpt.EE6 (6.652 kb) vector are illustrated above. Heavy bars on the plasmid indicate cloning site with REs used to excise the insert indicated. Numbers before other REs indicate cut site on plasmid.

Lck

The presence of an insert in the plasmid was determined by digestion with EcoRI or HindIII. Both of these had a unique restriction site in the lck insert rather than the vector. The expression vector alone without insert was 6.65 kb long and with lck cDNA was 8.20 kb long (fig 3.1b). Digests of unique sites, therefore, by either EcoRI or HindIII should and did yield a linear 8.2 kb fragment.

To analyze the direction in which the cDNA insert lay in the vector, it would be useful to find a restriction site within the insert which was considerably asymmetric with respect to the length of the site i.e. more 5' than 3' or vice versa. To perform a single digest, thereby avoiding complications in digest efficiency by using non-optimal buffers/ conditions for different restriction enzymes, it would also be useful to have a second site of the same enzyme in the vector as well as the insert. The BamHI site served this purpose and if the cDNA was oriented in the sense direction i.e. read 5' to 3' with respect to the direction of the promoter activity, then it was predicted that 486 b and 7.71 kb fragments would be produced. If in the anti-sense version, the expected products would be 1.526 kb and 6.676 kb long.

Figure 3.1a (lane 2) showed a sub-8.2 kb fragment (which could be either sense or anti-sense) and an absence of any other discernible band. Critically, it was the absence of the 1.526 kb band which indicated that lck was oriented correctly. If this band had been present it would indicate, that an anti-sense cDNA insertion was present. Its absence was not due to assay insensitivity, as a digest performed on fyn vector yielded a 1.6 kb product which was easily visible (see lane 5). In fact, although it is hard to see, there was a band of ~ 0.5 b which was visible to the eye (in the lck BamHI digest), although rather weak in intensity. As signal strength is proportional to the mass of DNA present it was not surprising that the smaller band was weak in signal intensity.

The undigested plasmid showed rather an intense signal which was not well resolved. This could be due to well overloading and so an incapacity of the agarose gel to properly sieve such a relatively high mass of DNA.

Fyn

To check the fyn expressing plasmid for correct size, a unique restriction site was found that could be cut by the restriction enzyme TthIII I (fig. 3.1b). Figure 3.1a illustrated two points about the plasmid. Namely a linear plasmid of predicted 8.274 kb could be detected from this digest. And as the restriction site lay in the insert rather than the plasmid, there was an insert within the plasmid. Unlike the lck vector, the fyn vector had two HindIII sites flanking the insert. Therefore a HindIII digestion with of the fyn vector would reveal a 1.6 kb fyn insert sized fragment and a 6.7 kb plasmid sized fragment. This was indeed the case.

To orientate the insert a digest was performed with ApaI which lies acentrically within the fyn cDNA insert and has one other site within the plasmid. The predicted fragment sizes were;

	sense	2.578 kb and 5.696 kb
	antisense	2.282 kb and 5.992 kb

From the markers used, it was possible to see that the critical determinant would have been the smaller fragment which if the cDNA was oriented in a sense fashion would lie above the level of the 2.3 kb marker and vice versa for the anti-sense version. Lane 7 (fig. 3.1a) showed fyn to be oriented in sense, as the lower marker lies above the level of the 2.3 kb marker with a position that could correspond to a 2.6 kb fragment.

Therefore, it appeared that the plasmids were correctly oriented to express the PTKs and were available, in sufficient quantities, to attempt transfection of $CD28^{+}$ve CHO cells.

3.2 Detection of Fyn, Lck and ZAP70 in $CD28^{+}$CHO Cells

To establish cell lines expressing one of the PTKs involved in CD28 signalling, $CD28^{+}$CHO cells were transfected with the PTK vectors. Following transfection the populations were expanded under XMAT selection. Detection of fyn, lck and ZAP70 in the transfectants was determined by Western blotting (fig 3.2a-c). Primary and secondary antibodies were titrated to determine optimum dilutions for specific detection

of the PTKs without contributions from non-specific molecules which may cross react with the blotting antibodies. In the detection of lck (fig. 3.2b) and ZAP70 (fig. 3.2c) many non-specific bands appeared when either the primary or secondary antibody concentration was increased. In the case of lck two bands between 56 and 60 kDa were detected. This is consistent with a reported retardation in electrophoretic mobility of activated lck, whereby the inactive form may be seen at 56 kDa and the activated form at 60 kDa (August and Dupont, 1994a). It was difficult to say with any certainty whether the upper band was indeed an lck gene product as a similar band was apparent in the CHO control at some dilutions. ZAP70 (fig. 3.2c) and fyn (fig. 3.2a) showed transfectant specific products migrating at approximately 70 and 59 kDa respectively. These results are consistent with previous reports detailing the migration of fyn, lck and ZAP70 under SDS-PAGE at respectively 59, 56-60 and 70 kDa (Mustelin, 1994). A compromise between strength of signal and specificity was reached by using antibody dilutions at:

Protein Detected	Exposure	Primary Dilution	Secondary Dilution
Fyn	3 mins	1/ 500	1/ 30000
Lck	3 mins	1/ 200	1/ 30000
ZAP7070	30 secs	1/ 1000	1/ 30000

In subsequent blots for fyn, lck or ZAP70, the conditions listed in the above table were used to determine levels of protein expression.

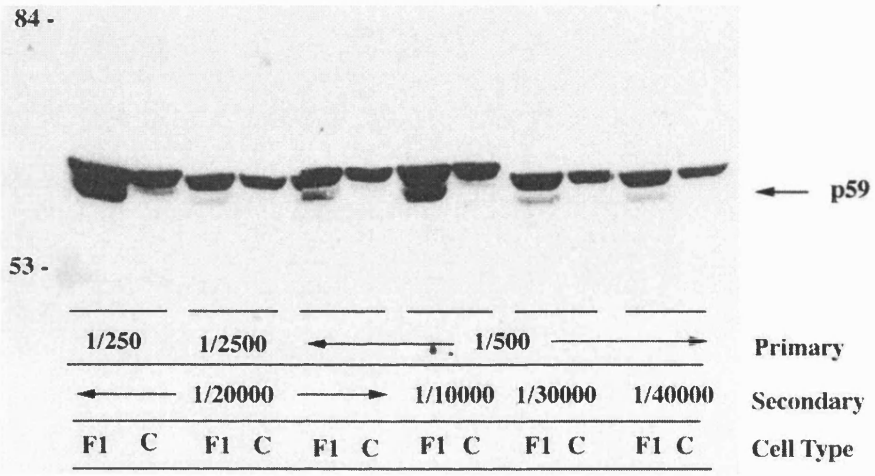


Fig. 3.2a: Optimization of Fyn Detection in Fyn Transfectants

$CD28^{+}$CHO cells were transfected with a vector expressing Fyn cDNA. Following expansion, $1x10^{6}$ Fyn transfectants (F1) or CHOs (C)/ lane were lysed and analyzed by SDS-PAGE separation, transfer to a membrane and Western blotting. Primary (rabbit derived αFyn polyclonal antibody) and secondary (goat α-rabbit antibody) antibody concentrations were titrated to determine optimum signal detection. Arrow indicates position of $p59^{fyn}$. A representative experiment of n=3 is shown.

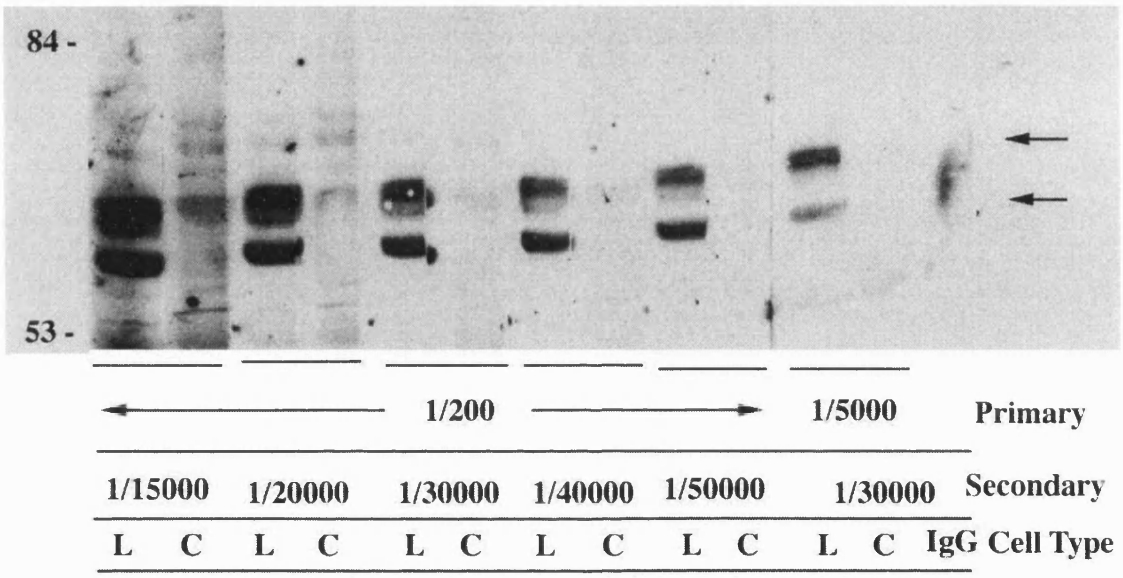


Fig. 3.2b: Optimization of Lck Detection in Lck Transfectants

$CD28^{+}$CHO cells were transfected with a vector expressing lck cDNA. Following expansion $1x10^{6}$ lck transfectants (L) and CHOs (C)/ lane were lysed and analyzed by SDS-PAGE separation, transfer to a membrane and Western blotting. Primary (rabbit derived αLck polyclonal antibody) and secondary (goat α-rabbit antibody) antibody concentrations were titrated to determine optimum signal detection. Arrows indicate positions of putatively activated $p60^{lck}$ (upper) and inactive $p56^{lck}$ (lower). A representative experiment of n=3 is shown.

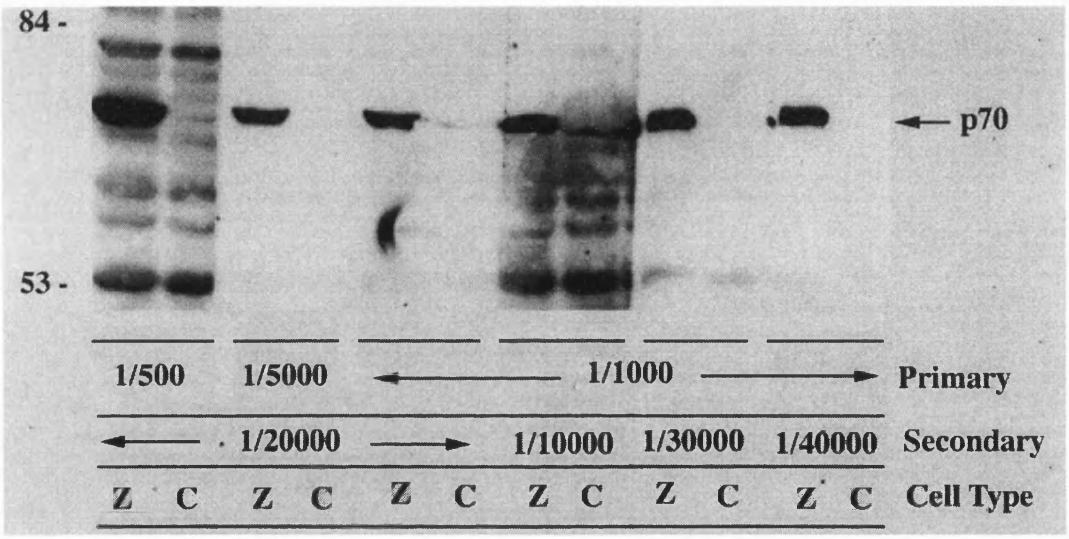


Fig. 3.2c: Optimization of ZAP Detection in ZAP Transfectants

$CD28^{+}$CHO cells were transfected with a vector expressing ZAP cDNA. Following expansion $1x10^{6}$ ZAP transfectants (Z) and CHOs (C)/ lane were lysed and analyzed by SDS-PAGE separation, transfer to a membrane and Western blotting. Primary (rabbit derived αZAP polyclonal antibody) and secondary (goat α-rabbit antibody) antibody concentrations were titrated to determine optimum signal detection. Arrow indicates position of $p70^{ZAP}$. A representative experiment of n=3 is shown.

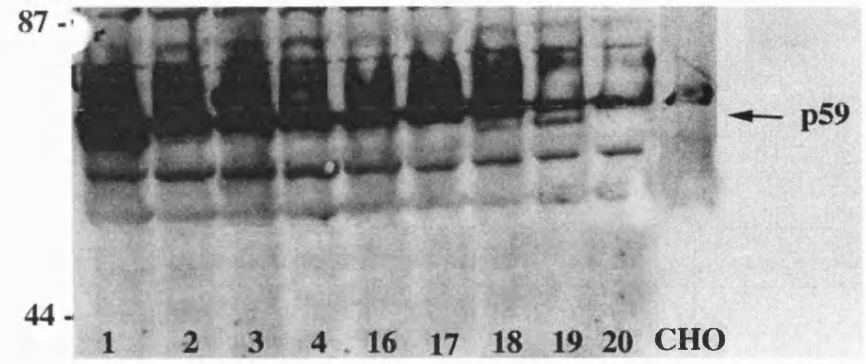
3.3 Expression Levels of Fyn and Lck Protein in Clones

While clonal populations of cells facilitate experimental reproducibility, achieving clonal populations of cells with the desired characteristics may be difficult. However to assess the contributions of PTKs, without variation arising from a polyclonal population, transfectants were screened by Western blotting in order to attempt the identification of clones with detectable and preferably high levels of PTK expression. Initially the PTK transfected $CD28^{+}$CHOs were stained with a primary αCD28 mAb and secondary FITC conjugated antibody for FACS analysis. The cells which had homogeneously high expression of CD28 were directed in to a 96 well microtitre plate by a Becton Dickinson ACDU application at 1 cell/ well for 20 clones. Subsequently these were expanded under XMAT selection before analysis by Western blotting.

Fyn transfectant clones showed (fig. 3.3a) that only clone 1 (F1CC) had relatively high expression of fyn with clones 18 and 19 showing some fyn expression although at a lower level. Lck transfected clones did not show (fig. 3.3b) detectable lck expression although the uncloned "LCC" populations did. Due to the limited number of fyn transfectants and the absence of clonal lck transfectants expressing easily detectable levels of PTKs, further attempts to isolate PTK clones were not performed.

In conclusion F1CC, LCC and $Zap^{+}CD28^{+}$CHO (ZCC uncloned) cell populations were deemed to have sufficiently high levels of PTK expression to differentiate them from the parental $CD28^{+}$ve CHO cell line and so were used in subsequent experiments.

a



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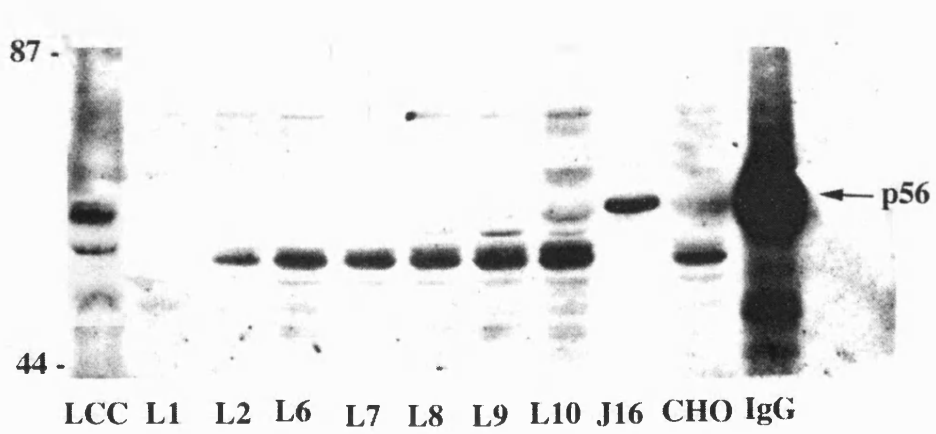


Fig. 3.3: Expression Levels of a) Fyn and b) Lck in Transfected CD28^{+}ve CHO Clones

Above is representative example of twenty CD28^{+}CHO clones transfected with a) fyn or b) lck expression vectors which were expanded and Western blotted for levels of fyn and lck protein expression. 1x10^{6} cell equivalents/ lane were analyzed. The arrows in a and b represent respectively the migration positions of p59^{fyn} andp56^{lck}. A representative sample of n=2 is shown.

3.4 Development of a $CD28^{LOW}$ J16 line

In order to develop a better understanding of the role of CD28 and the signals controlling costimulation in T cells, a T cell line which could act as a parental cell host for CD28 intracellular domain mutants was developed. The Jurkat subclone, J16, was subjected to various treatments to deplete the population of CD28 expressing cells. The first type of treatment was by labelling the cells with αCD28 mAb followed by complement mediated lysis. Following recovery of live cells and their expansion the process was repeated four times. Figure 3.4 shows CD28 expression in the parental line J16 and after the fourth round of complement treatment, where the cell type is labelled JcR4. Following expansion the population of cells were labelled again with αCD28 mAb and incubated with magnetic beads carrying anti-mouse immunoglobulin. Then the population was exposed to a magnet to deplete the population of CD28 positive cells further (fig. 3.4, JMAG). Finally the population of cells not removed by the magnet were expanded and in two consecutive rounds sorted by FACS for cells showing a comparable level of fluorescence (FL1) to the negative control. FL1 was proportional to the level of CD28 expression and the negative control had not been labelled with αCD28 antibody. The level of CD28 expression following the second round of selection of CD28-ve cells by FACS using an ACDU programme, labelled 28N (for CD28 Negative J16s), is illustrated in figure 3.4. A marked decrease from 98.9% to 24.6% of the population showed CD28 expression between the J16 and 28N cell lines.

3.5 Phenotype of a $CD28^{LOW}$ Jurkat Cell Line

To assess whether or not the strategy which depleted CD28 from the cell surface of Jurkats was specific to depleting CD28, the parental cell line, J16, and the $CD28^{LOW}$ line, 28N, were assessed for the level of other surface markers. The cells were stained for FACS analysis with antibodies against a range of membrane proteins. The overlapping histograms in figure 3.5 allow a comparison of the level of expression between J16 and 28N for the surface markers. In fact the two cell lines had comparable expression of CD2, CD3, CD4, CD7, CD8, CD25, CD48 and LFA-3. The only marked difference between the two populations was the level of expression of CD28 which was far lower in 28N than in J16.

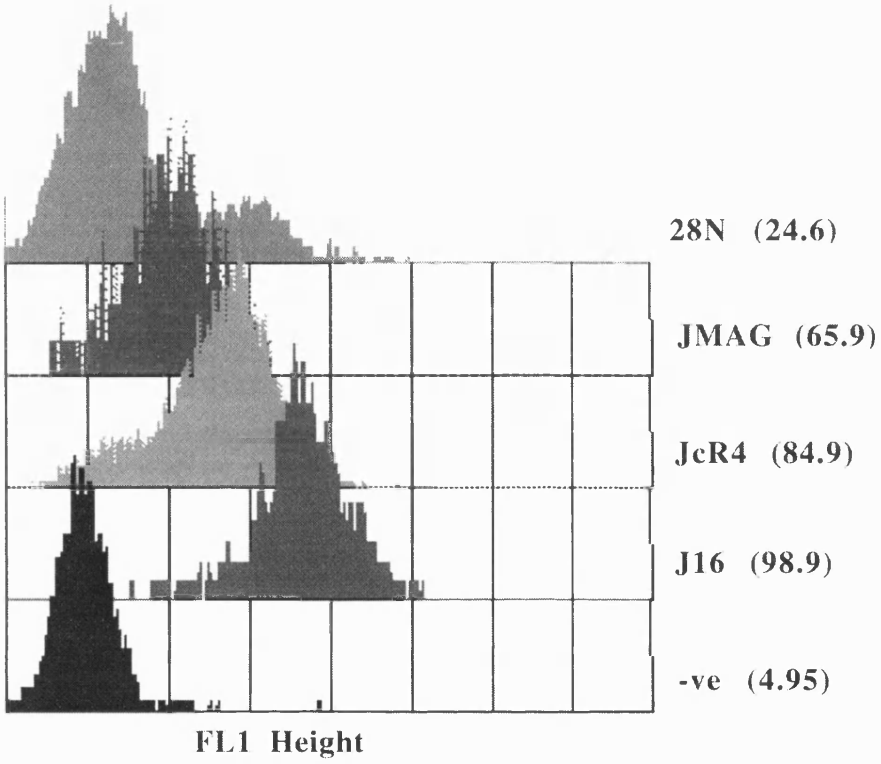


Fig. 3.4: Generation of a CD28$^{-ve/\ Low}$ T cell Line.

αCD28 mAb labelling of cells followed by complement mediated lysis (JcR4), magnetic depletion of cells binding αCD28 (JMAG) and selection of CD28 -ve cells by FACS (28N) generated a CD28 low T cell line, 28N, from J16 Jurkats. A histogram shows the level of CD28 expression assessed by FACS at each stage of treatment. The percentage of cells showing fluorescence above the negative control is indicated in brackets.

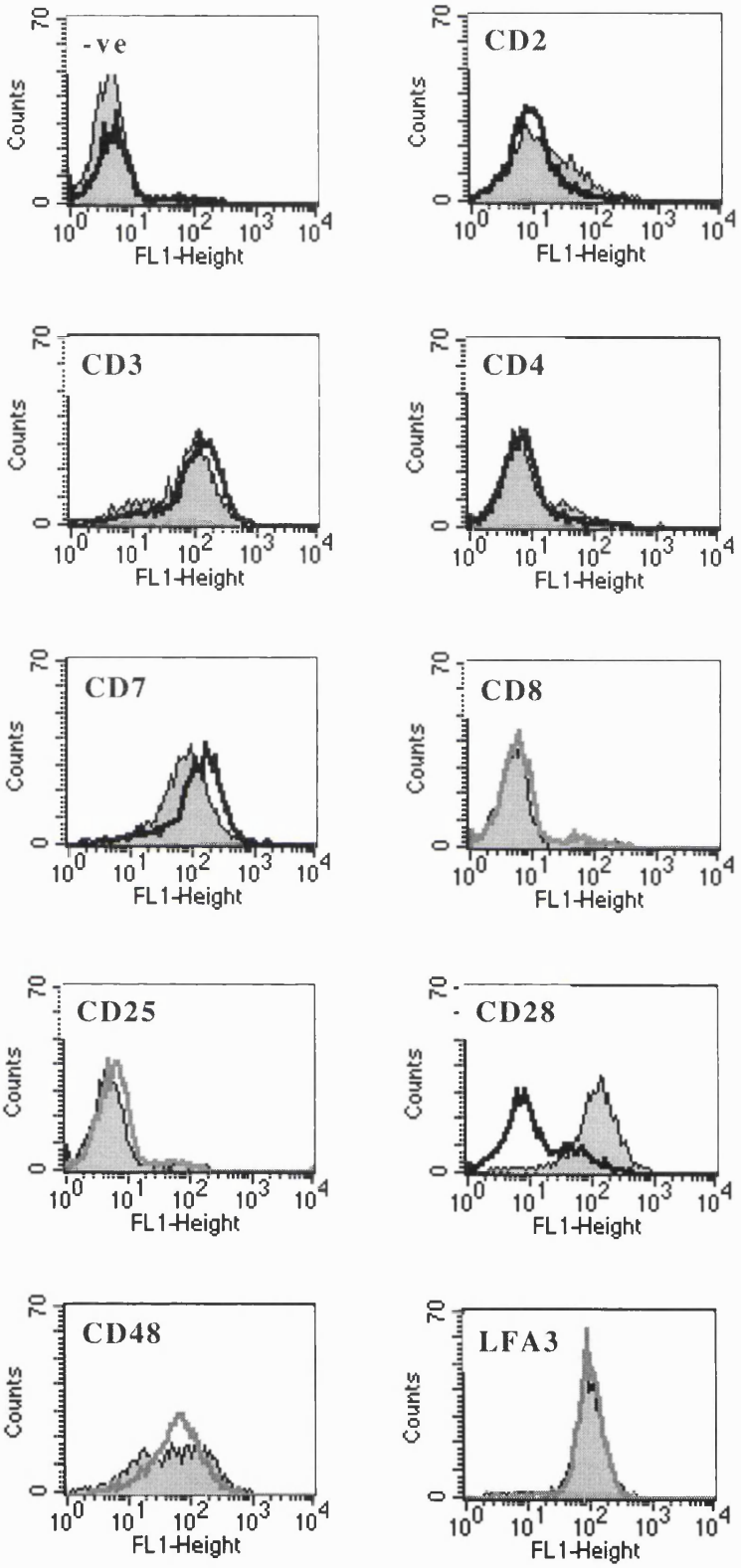


Fig. 3.5: Comparison of Surface Markers between J16 and a CD28^{LOW} J16 Line, 28N

2x10^5 J16s (filled grey histograms) or 28Ns (lines) were fluorescent stained for surface markers and anayzed by FACs. Overlapping histograms or J16 and 28N fluorescence are shown.

3.6 Discussion

To analyze the role of PTKs in CD28 signalling, a number of cell lines were developed. $PTK^{+}CD28^{+}CHO$ cells would facilitate an analysis of different effects between one PTK and another in the phosphorylation of CD28 or associated substrates of CD28. In addition PTK transfectants would be useful in analyzing the possible contribution PTKs make in the recruitment of other signalling molecules to CD28. The Jurkat $CD28^{LOW}$ cell line, 28N, might be useful in assessing the role of CD28 cytoplasmic mutants or CTLA4 if they were transfected into 28N.

The use of clonal populations in biological responses facilitates reproducible results due to the homogeneity of biochemical signalling within such a population compared to a mixed population. In this chapter, 20 clones of fyn and lck $CD28^{+}CHO$ transfectants were screened by Western blotting following their expansion under selection. It was noted that one clone of the fyn transfectants expressed markedly greater levels of fyn protein and two others expressed moderate amounts of fyn. In contrast, of the lck clones tested, none expressed a level of lck detectable by Western blotting. Thus the non-clonal population (LCC) was designated as the more useful cell line for future experiments. Due to the lack of a high or even detectable level of lck in the clonal populations, it was decided that as the non-clonal ZAP70 population expressed clearly detectable levels of protein specific to the ZAP70 gene, that it might be excessively costly in time to attempt to find a clone which may/ not express greater levels of ZAP70. And so a non-clonal population, ZCC, was used in analysis of the function of ZAP70 in CD28 signalling.

It is interesting to note that despite expansion of newly transfected cells and later clones under selection none of the lck clones and few of the fyn clones expressed detectable PTK. Therefore while the cells had to express the gpt gene to proliferate under XMAT selection (Mulligan and Berg, 1981), it was apparent that the expression of a PTK cDNA insert was not necessarily concomitant with expression of a selectable marker gene.

To assess the level of protein expression from transfected genes Western blotting was employed. The primary antibody, raised against the gene product and the secondary antibody, which had the primary antibody as a substrate, determined the specificity of Western blotting. Crossreactive molecules to primary antibodies induce the appearance of bands in Western blots which are not specific to the expressed transfected gene product. The secondary antibody functions to regulate the level of signal. Therefore where specificity is problematic, provided that the target signal is the most intense, primary and/or secondary antibody concentrations may be lowered until only the strongest signal is visible. Further the length of time a film is exposed to signal (from the enhanced chemiluminescent source of emission) determined signal strength, and apparent specificity, in a manner analogous to the secondary antibody.

In order to attempt further analysis of CD28 signals and provide information on the type of interaction(s) transducing costimulatory signals, a $CD28^{LOW}$ T cell line was developed. This $CD28^{LOW}$ T cell was intended to be a host for subsequent transfection of CD28 cytoplasmic mutants. Alternatively CTLA4 might be transfected into the cell line. A variety of methods were used to develop a CD28 -ve T cell line, all based upon deletion of cells reactive to αCD28 mAb from the population. A cell line with markedly lower CD28 expression was achieved and denoted 28N. However throughout the CD28 deletion strategy, it became apparent that the reductions in CD28 surface expression were not necessarily permanent and given time may return, perhaps to levels comparable to the parental line, J16. Therefore after expansion of the 28N population and checking that CD28 expression was still low, a number of vials were cryopreserved and the remainder were further expanded and rapidly used. Phenotypically 28N had slight differences from J16 in CD48 and CD2 expression and except for the large difference in CD28 expression, both populations had comparable expression of other surface markers. Whether or not differences in response of 28N arise solely from its lower expression of CD28, rather than small changes in CD48 and CD2 was not investigated. Due to the partial nature of the difference in expression between 28Ns and J16s of these latter two surface markers, it would seem more likely that differences in response between the two cell lines would

arise from the distinctly lower surface expression of CD28 on 28N compared to those of CD28 on J16.

In conclusion a number of cell lines of the required phenotype were successfully generated. For example the clonal $fyn^{+}CD28^{+}CHO$ cell line, F1CC, expressed detectable levels of fyn and CD28, the uncloned transfectants LCC and ZCC expressed detectable levels of lck and ZAP70 respectively as well as CD28, while 28N had depleted levels of CD28. Further, a Western blotting assay had been optimized such that specific detection of fyn, lck or ZAP70 expression in the PTK was possible. Monitoring of the level of CD28 expression in the cell line 28N was performed by FACS. Therefore it was possible to conclude that the cellular models required were ready for use and so assays were devised to assess the effect of ligating CD28, by one of its natural ligands, CD80.

Chapter 4

Analysis of Proximal Signals of CD28

The mechanism by which CD28 transduces costimulatory signals is not known despite growing interest in the field. Studies attempting to identify proximal signalling molecules involved in CD28 costimulatory pathways are obscured by a number of issues. For example the action of an inhibitor herbimycin A in ablating CD28 costimulation of IL2 production (Vandenberghe et al., 1992; Lu et al., 1994) has lead to the belief that protein tyrosine kinases are important in transducing CD28 proximal costimulatory signals. However because herbimycin A is directed against sulphydryl (SH) moieties (Uehara et al., 1989), it may be suggested that it lacks specificity. Therefore the role of PTKs in CD28 signalling remains to be investigated. Interpretation of the results of other studies addressing the contribution of PTKs by analysis of PTK mutant cell lines e.g. JCaM1, which is lck negative, also presents difficulties because T cells possess a number of PTKs from the src, syk and EMT families and these may have functional redundancy. For example Lu et al demonstrated phosphorylation of a p110 protein with and without contributions from lck in Jurkats and JCaM1s respectively (Lu et al., 1994) following CD28 ligation. Thus it is difficult to determine whether lck was the kinase capable of phosphorylating p110 in the lck^{+ve} Jurkat or whether an alternative PTK had substituted the role of lck. An alternative method to study the contribution of individual elements of CD28 signalling is needed. One theoretical possibility would be to transfect resting T cells with kinase inactive PTKs and determine which combination of these regulates CD28 signalling. However this is not yet possible due to difficulties in resting T cell transfection whereupon they show low survival rates. Transfection of activated T cells or Jurkats is possible e.g. with IL2 promoter constructs (Stein et al., 1994; Boucher et al., 1995; Crooks et al., 1995) but may not supply accurate information on the costimulatory requirements of resting T cells due to differences in biochemical signalling pathways from resting T cells. For example inhibition of PI3K activity by incubation of resting T cells in wortmannin inhibits IL2 production despite costimulation, but wortmannin has no effect or possibly augments IL2 production in Jurkats and T cell blasts (Ward et al., 1995; Ueda et al., 1995). Therefore the use of "activated" T cells in studies attempting to analyze the transduction pathways arising from CD28 in resting T cells may be of limited use.

This chapter attempted to overcome some of these problems and in order to analyze interactions with CD28 during signal transduction, individual PTKs were expressed in CHO cells expressing CD28 ($CD28^+$CHOs) and assessed for their contribution to CD28 signalling. We analyzed immunoprecipitates of CD28 for tyrosine (tyr), serine (ser) and threonine (thr) phosphorylation changes in response to stimulation with and without CD80. Attempts were also made to analyze the effect of lck on PI3K recruitment to CD28 and in addition due to reports that CD28 costimulatory function may be replaced by ASMase in murine splenic T cells (Boucher et al., 1995; Chan and Ochi, 1995), CD28 was examined for its capability to stimulate ASMase.

4.1 Immunoprecipitation of CD28

In order to determine the effect of ligating CD28 in the absence of contributory signals from other receptors and to optimize the sensitivity of detection of CD28-associated signals, CD28 was immunoprecipitated from CHO transfectants. In order to verify the procedure and due to a paucity of αCD28 antibodies suitable for Western blotting, CD28 and other cell surface proteins were initially labelled with biotin. Labelled $CD28^+$CHOs or control CHOs were lysed, incubated with αCD28: Protein A-Sepharose, subjected to SDS-PAGE and Western blotted with an HRP.avidin D conjugate directed against biotin and the products were visualized using ECL reagents. This process yielded a broad band of approximately 44-54 kDa from $CD28^+$CHOs, but not from non-transfected CHO cells (fig. 4.1). A second band at ~70 kDa of weaker intensity was also apparent and this may be an associated surface protein or a partially reduced CD28 dimer. The nature of this high molecular weight molecule was not investigated further although it is unlikely that it was a CD28 dimer due to the molecular weight which was not consistent with an expected molecular weight of 88 to 108 kDa for a CD28 dimer. Thus, as a result of transfecting CHO cells with CD28, a product of 44-54 kDa could be immunoprecipitated from these cells. This is in agreement with previous findings that CD28, while predicted to be expressed as a protein of molecular weight 23 kDa from the gene sequence (Aruffo and Seed, 1987) becomes heavily glycosylated (Aruffo and Seed, 1987; Hara et al., 1985) accounting for the breadth of the band immunoprecipitated.

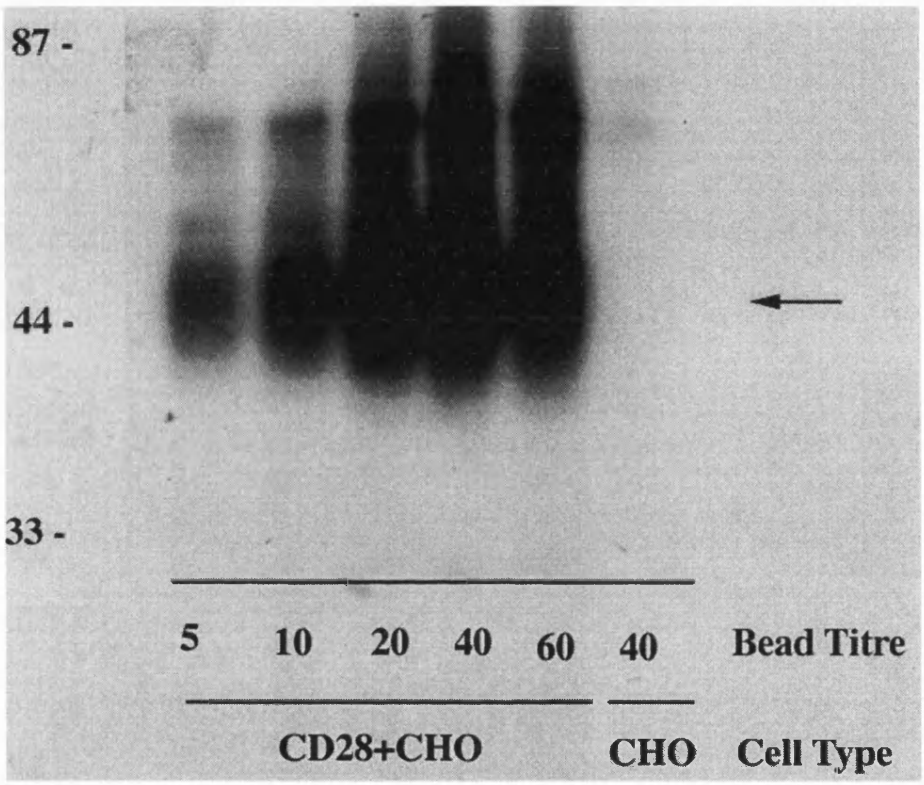


Fig. 4.1: A Broad 44 - 54 kDa Band Can Be Immunoprecipitated from $CD28^{+}CHO$ Cells But Not from Parental CHO Cells

Precoupled αCD28: Protein A Sepharose beads immunoprecipitated a broad band of ~ 44-54 kDa from biotinylated cells transfected with CD28, but not where cells did not express CD28. 5-60µl of αCD28: Protein A Sepharose beads were titrated against 5 million cells/ lane, CD28 was immunoprecipitated and Western blotted with Streptavidin. HRP. A representative experiment of n=3 is shown.

To optimize the sensitivity of immunoprecipitates, a titration of αCD28: Protein A beads against a fixed number (5 million) of biotinylated $CD28^{+}$CHO cells was performed. The optimum ratio of antibody precoupled beads to cell number was determined from experiments such as this and in the preparation used in figure 4.1 was 40μl of beads for 5 million cells. Thus for subsequent experiments, depending on the cell number required, the volume of beads could be altered to maximize signals associated with CD28.

4.2 Establishing a Tyrosine Phosphorylation Assay

In order to establish an assay to detect tyrosine phosphorylation by Western blotting which could subsequently be used to assess the tyrosine phosphorylation status of CD28, changes in tyrosine phosphorylation occurring after ligation of CD3 were assessed, as CD3 has been reported to undergo tyrosine phosphorylation upon stimulation (Rudd et al., 1994; Straus and Weiss, 1993; Irving and Weiss, 1991). Lysates from 1 million Jurkats, stimulated by αCD3 mAb and lysed after incubation periods between 0 and 30 mins, were assessed for changes in tyrosine phosphorylated proteins on the Western blotted lysates. It was found that in Jurkats there was some constitutive tyrosine phosphorylation (fig. 4.2) of ~29, 35, 44, 53, 63, 67, 85, 90 and 120 kDa proteins. Stimulation through CD3 rapidly increased both the overall level of tyrosine phosphorylation and induced the appearance of specific tyrosine phosphoproteins at ~23 kDa and between 46 and 120 kDa. In addition some phosphotyrosine proteins disappeared during stimulation e.g. an 85 kDa protein. An increase in tyrosine phosphorylation was apparent 10 seconds after stimulation of CD3, peaking at 1-2 minutes. Thereafter a gradual decline was evident although there was still a greater level of tyrosine phosphorylation at 30 minutes post-stimulation than in the resting Jurkats. The tyrosine phosphorylation profile observed from Jurkat lysates following CD3 stimulation concurs with that observed in other reports (Irving and Weiss, 1991). These tyrosine phosphorylated molecules represent substrates of tyrosine kinases associating with the TCR such as CD3 and TCRζ, kinases and adapter molecules facilitating TCR signal transduction. For example 23 kDa CD3ε and CD3δ have been reported to become tyrosine phosphorylated following TCR stimulation (Straus and Weiss, 1993) and the 40

kDa molecule may represent tubulin while the kinases fyn, lck and ZAP70 which are negatively regulated upon tyrosine phosphorylation by $p50^{csk}$ are of 59, 56-60 and 70 kDa respectively (McFarland et al., 1992). However the identity of these molecules was not further investigated because we were not trying to establish the identity of CD3-stimulated phosphotyrosine proteins and this experiment served to demonstrate that we were capable of detecting phosphotyrosine proteins by Western blotting. The changes in various phosphoproteins also served to establish that the detection was specific as stimulation through an irrelevant antibody (L243, directed against HLA-DR proteins, lane 9) did not show a comparable increase in tyrosine phosphorylation at 30 minutes post-stimulation compared to the level induced by the αCD3 mAb (lane 8) . In conclusion we established an assay capable of measuring changes in tyrosine phosphorylation which could then be used in CD28 assays.

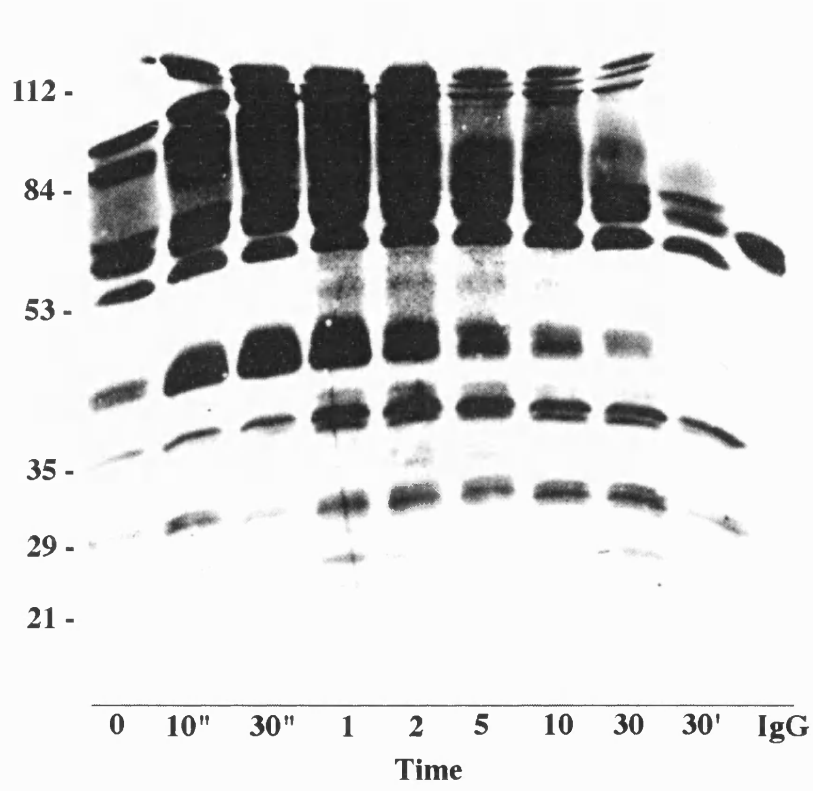


Fig. 4.2: Ligated CD3 Modulates Tyrosine Phosphorylation in Jurkats

$1x10^6$ Jurkats/ lane were stimulated with αCD3 mAb (10μg/ ml) for the indicated time periods at 37°C where 10" and 30" referred to seconds and other time points were in minutes. The point 30' was stimulated by an irrelevant antibody. Following stimulation, lysates were analyzed for changes in tyrosine phosphorylation by Western blotting with 4G10. A representative experiment of n=3 is shown.

4.3 The Effect of Fyn, Lck and ZAP70 on Tyrosine Phosphorylation of CD28 Immunoprecipitates

We next attempted to examine the effect of the PTKs fyn, lck and ZAP70 on modulating the tyrosine phosphorylation of CD28 and associated molecules following stimulation by CD80 (B7). CD28$^+$CHO cells were stimulated with/ without CD80, CD28 was immunoprecipitated from lysates and following SDS-PAGE, Western blotted with the primary mAb 4G10 for phosphotyrosine proteins. Figure 4.3 illustrates that the levels of tyrosine phosphorylation associated with CD28 immunoprecipitates were generally greater in the presence of a PTK transfected into CD28$^+$CHO cells, although none of the increases in tyrosine phosphorylation appeared specific to the ligation of CD28. Indeed the increases in tyrosine phosphorylation in the presence of PTKs did not appear specific either in that most of the substrates phosphorylated appeared to be present in CD28$^+$CHOs, albeit tyrosine phosphorylated to a lower extent . Jurkats showed similar tyrosine phosphorylation patterns to PTK$^+$CD28$^+$CHO cells. It would appear then that little/ no increase in CD28 tyrosine phosphorylation is facilitated without the presence of additional signals e.g. PTKs in agreement with the lack of an intrinsic PTK activity of CD28 (Aruffo and Seed, 1987).

In the presence of T cell PTKs, irrespective of stimulation with or without CD80, there was an overall higher level of tyrosine phosphorylation associated with CD28 immunoprecipitates. Previous reports have described the induction of phosphotyrosine proteins such as the T cell expressed p100^{Vav} (Nunes et al., 1994) and p62, an adapter protein (Nunes et al., 1996) following CD80 stimulation of CD28. There was no evidence that a 100 kDa band was induced in our experiments in Jurkats although it may be possible that a 62 kDa protein was present in the fyn transfectant immunoprecipitates independent of CD80-CD28 interaction. However due to strong signals from the reduced heavy chain immunoglobulin band at ~55 kDa, it is difficult to decipher whether the 62 kDa protein is part of the immunoglobulin band or present due to fyn activity. Despite the lack of inducible changes in tyrosine phosphorylation recorded in CD28 immunoprecipitates, Jurkat lysates (lane 13) stimulated with an αCD3 mAb (OKT3) demonstrated that tyrosine phosphorylation could be observed. Overall the results from

these experiments were disappointing and did not reveal specific, kinase-dependent phosphotyrosine changes in CD28 immunoprecipitates.

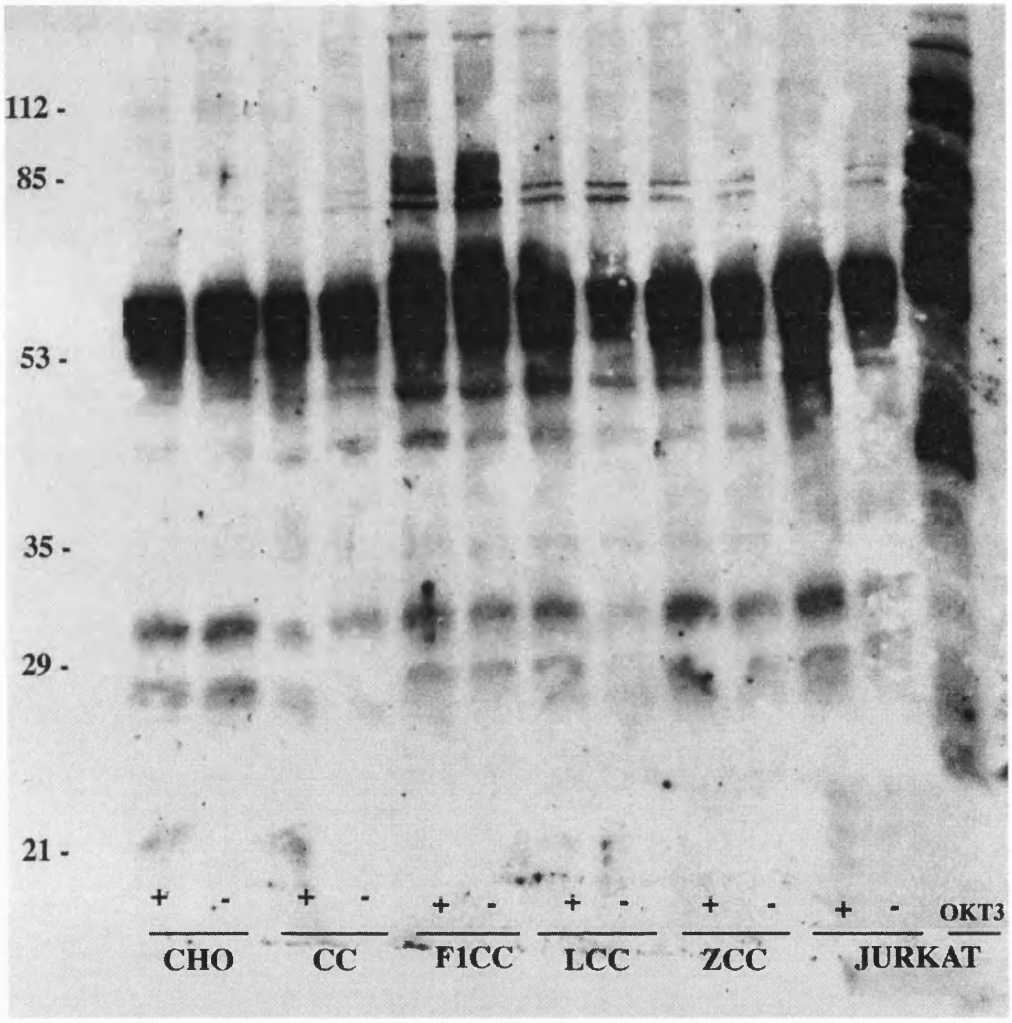


Fig. 4.3: Effect of Fyn, Lck or ZAP on Tyrosine Phosphorylation of CD28 Immunoprecipitates +/- CD80 Stimulation

$2.5x10^7$ cells/ lane were stimulated with (+) $CD80^+CHO$ or with CHO (-) at a ratio of 3:1 for 5 mins at 37°C. Following immunoprecipitation with αCD28:Protein A beads, changes in tyrosine phosphorylation were assessed by SDS-PAGE separation and Western blotting. CHO, Chinese hamster ovary; CC, $CD28^+CHO$; F1CC, fyn^+CC (clone 1); LCC, lck^+CC; ZCC, ZAP^+CC; $1x10^6$ Jurkats were stimulated with OKT3 (10µg/ ml as a positive control) for 2 minutes and the lysate was analyzed. A representative experiment of n=3 is shown.

4.4a The Effect of Fyn, Lck and ZAP70 on the Phosphorylation of CD28 Immunoprecipitates

We next examined whether fyn, lck or ZAP70 could either facilitate the association of other kinases with CD28 or functionally associate themselves with CD28. $CD28^+$CHOs, Jurkats, CHOs or PTK transfected $CD28^+$CHOs were stimulated for 5 mins at 37°C with or without CD80 and PTK-modulated phosphorylation of CD80-stimulated CD28 was assessed. CD28 was immunoprecipitated and subjected to a [^{32}P γ] ATP in vitro kinase (IVK) reaction. The reaction products were separated by SDS-PAGE and the dried gel was used to visualize the products which had incorporated ^{32}P γ by autoradiography. Interestingly, both fyn and lck specifically enhanced phosphorylation of CD28 immunoprecipitates in response to CD80-CD28 interaction (fig. 4.4a), a result which contrasted with that observed in analysis of tyrosine phosphorylation changes (fig. 4.3). Three substrates in lck or fyn transfectants (LCC or F1CC) appeared to show increased levels of phosphorylation and these migrated at approximately 44, 85 and 110 kDa. It was found that lck and fyn transfectants but not ZAP70 transfectants were capable of demonstrating a CD80-induced increase in phosphorylation of CD28 immunoprecipitates (fig 4.4a). In particular the result for lck transfectants when stimulated by CD80 was most marked. Strikingly, Jurkats showed a similar pattern of phosphoprotein banding to that seen with lck and fyn transfectants. In addition in Jurkat cells, two bands at 56-60 kDa, which were not apparent in CHO transfectants, underwent a CD80-induced increase in phosphorylation. These may have represented lck because upon activation lck becomes more heavily phosphorylated and appears as a pair of protein bands at 56 and 60 kDa (August and Dupont, 1994a). For the ZAP70 transfectants (ZCC) there was no increase in either constitutive or CD80-induced levels of phosphorylation beyond that seen in the parental cell line $CD28^+$CHO. Thus the presence of ZAP70 did not facilitate increased phosphorylation of proteins associating with CD28. However, since direct precipitation of ZAP70 followed by assessment of kinase activity was not performed, we cannot at this stage exclude the possibility that the kinase was defective. Support for the hypothesis that fyn and lck but not ZAP70 mediate increased phosphorylation of CD28 immunoprecipitates could further be provided by a confirmation of an intact kinase activity of ZAP70. However the data suggest that fyn and lck, but not ZAP70, could

facilitate an increase in the phosphorylation of proteins corresponding to the molecular weights of CD28 and PI3K subunits, when CD28 was ligated by CD80, although the identity of these proteins is not proven. These data also concur with a report (Raab et al., 1995) which demonstrated lck and fyn, but not ZAP70, were sufficient to associate with CD28 immunoprecipitated *in vitro* kinase activity, although they did not address the effects of ligating CD28 with either ligands or antibody. In conclusion it would appear that fyn and lck are capable of associating with CD28 signal transduction and that fyn or lck either directly tyrosine phosphorylate CD28 and associated substrates or alternatively recruit ser/ thr kinases which phosphorylate CD28. Furthermore assuming equivalent levels of PTK expression in the cell lines, lck more than fyn had the greater capability to inducibly phosphorylation of CD28.

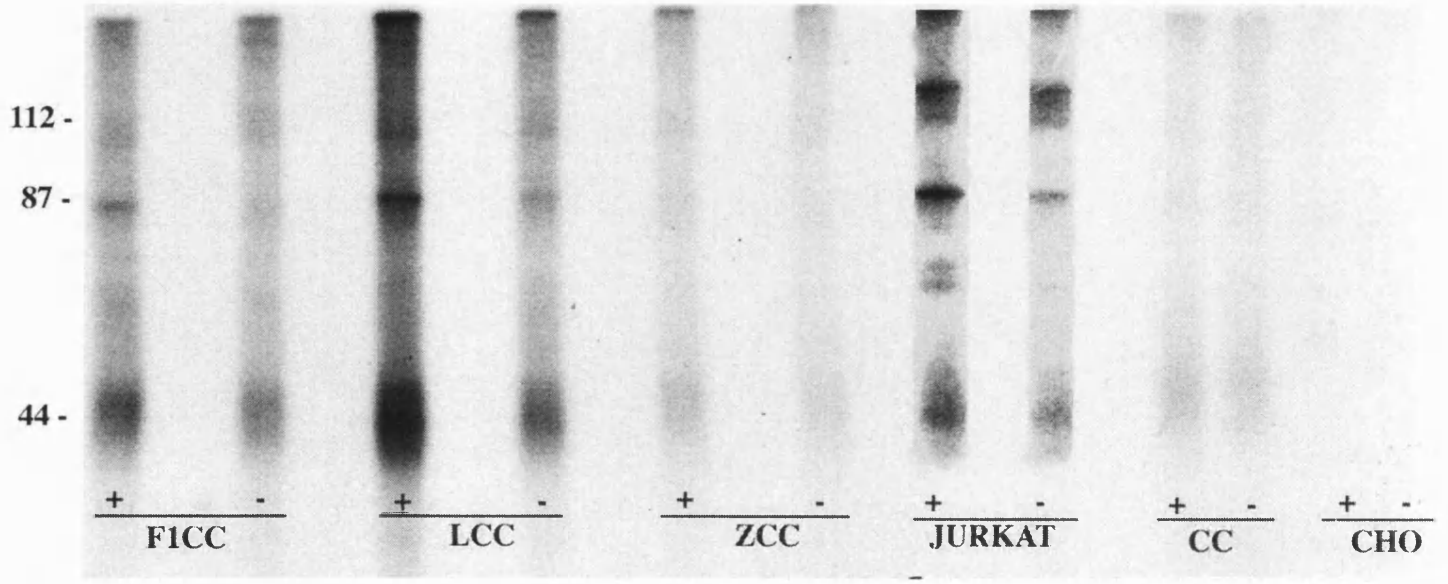


Fig. 4.4a: Effect of Fyn, Lck or ZAP on Phosphorylation of CD28 Immunoprecipitates +/- CD80 Stimulation

$1x10^7$ cells/ lane were stimulated with $CD80^+$CHOs (+) or CHOs (-) at a ratio of 3:1 responders to activators, for 5 mins at 37°C. CD28 was immunoprecipitated and IPs were subjected to IVK analysis. Cell lines as in figure 4.3. A representative experiment of n=3 is shown.

4.4b-c Fyn, Lck, ZAP70 and CD28 Expression in Cell Lines

In order to assess whether the differential phosphorylation of CD28 by the PTKs fyn, lck or ZAP70 may have been an artefact arising from variation in either PTK or CD28 expression, control experiments were performed to analyze their levels of expression.

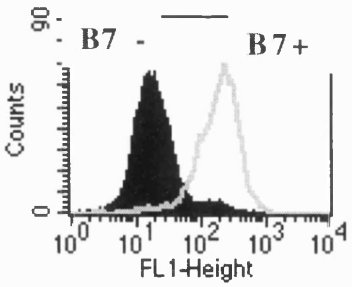
To analyze CD28 expression, FACS assessment of CD28 surface expression was employed. Figure 4.4b illustrated that CD28 expression was comparable between cell lines. Therefore the lower level of inducible changes seen with ZCC (compared to LCC and F1CC) could not be attributed to depleted levels of CD28.

To determine whether variation in PTK expression may have accounted for the difference in response shown in figures 4.3 and 4.4a, Western blotting was used to determine levels of PTK expression. Figure 4.4c demonstrated that PTK expression was heterogeneous between cell lines, relative to the level observed in Jurkats. When fyn expression was assessed, two bands at ~ 59 and 63 kDa were apparent in a blot of fyn transfectant lysates whereas only one band was present in the CHO control at ~ 63 kDa. Therefore the lower band at 59 kDa was specific to fyn and while this was apparent in the fyn transfectant, it was absent from the Jurkat lysate and CHO control. Therefore Jurkats did not express a protein migrating at the same molecular weight as the transfectant. However it is possible that fyn may be differently processed in Jurkats and therefore may migrate at a different rate under SDS-PAGE. Lck expression was markedly lower in the transfectants (LCC) than in Jurkats and ZAP70 expression appeared to be, like fyn, higher in transfectants than Jurkats. All transfectants showed gene products (indicated by arrow heads) at the expected molecular weights, respectively 59, 56 and 70 kDa for fyn, lck and ZAP70, which were clearly distinguishable from background cross reactive products. As ZAP70 and fyn expression in transfectants was relatively high compared to the levels observed in J16s, its was interesting that lck, expressed to a lower level in transfectants compared to J16s, should facilitate the most intense phosphorylation of CD28 associated substrates. It was therefore concluded that given the levels of expression as determined by Western blotting, lck was the most potent kinase tested for the recruitment of kinase activity to CD28 immunoprecipitates. The apparent lack of a role for ZAP70 is consistent with its

known role in TCR signalling and requirements for binding to CD3 and ζ subunits bearing an ITAM motif (Hatada et al., 1995) from the literature, however we were unable to rule out a defective kinase activity. Nonetheless these data strongly support a role for lck in recruiting p85/ p110 molecules to CD28 which are possibly PI3K, as well as causing the phosphorylation of a 44 kDa band which is probably CD28.

4.5 Deglycosylation of CD28

In order to attempt identification of the phosphoprotein migrating in the range 44-54 kDa which may have been CD28, immunoprecipitated CD28 was treated by a deglycosylating agent, PGNase F. While CD28 has a predicted molecular weight from nucleotide sequence data of 23 kDa (Aruffo and Seed, 1987) as a monomer, the mature protein has five potential N-linked glycosylation sites (Asn-Xaa-Ser/ Thr) (Aruffo and Seed, 1987). Thus as a mature protein CD28 under reducing conditions is reported to migrate as a broad band at ~44-54 kDa (Aruffo and Seed, 1987; Hara et al., 1985). Figure 4.5 shows that in $lck^{+}CD28^{+}$CHOs, stimulated by CD80, the phosphorylation state of a broad 44-54 kDa band, increased. Treatment with a deglycosylating agent, PGNase F, caused the disappearance of this band (top arrow) and the appearance of a 23 kDa band (bottom arrow) without affecting the migration rates of other phosphoproteins associated with CD28. Thus it seemed likely from this data and the information above that this broad 44-54 kDa band was CD28 and this concurred with data from a report (Hutchcroft et al., 1996) showing a shift in the electrophoretic mobility of deglycosylated CD28 from ~44 to 23 kDa.



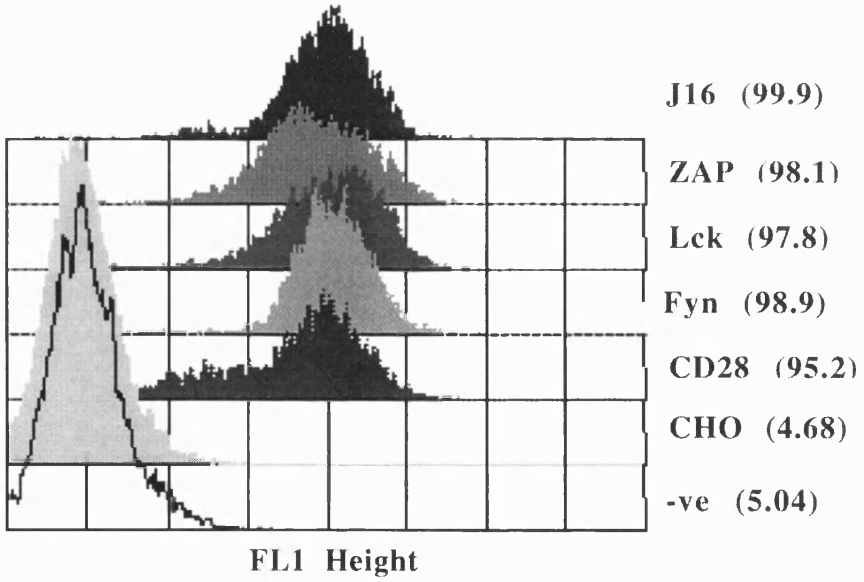


Fig. 4.4b: Levels of CD28 Surface Expression on Transfectants and Jurkats and B7 on $B7^+$CHOs

$2x10^5$ cells were fluorecent labelled with αCD28 (9.3) or αB7 (BB1) mAbs before FACS analysis. Cell samples analyzed were paired with the cell lines in fig 4.4a; fyn (FCC), lck (LCC), ZAP (ZCC), CD28 (CC) and J16 Jurkats. Figures in brackets indicate the percent of cells showing expression above 95% of the -ve control. CD80 (B7) expression relative to cells stained without the primary antibody is also shown in the top diagram.

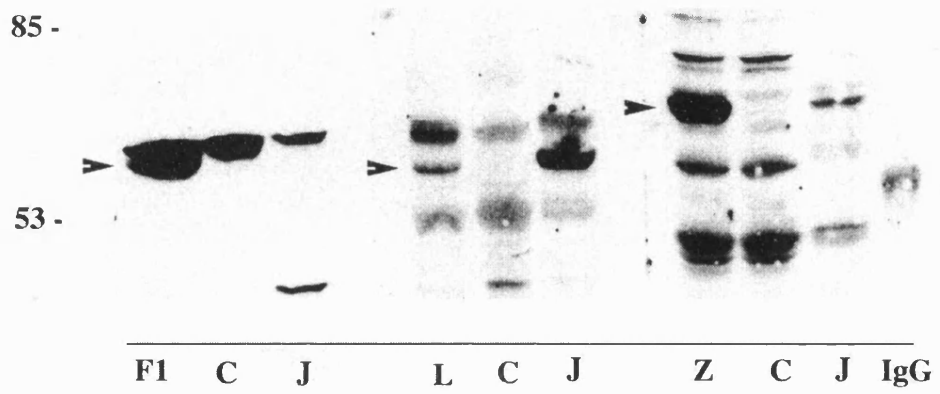


Fig. 4.4c: Levels of Fyn, Lck and ZAP in Transfectants and Jurkats

$1x10^6$ cells/ lane were lysed, their proteins separated by SDS-PAGE and Western blotted for fyn, lck or ZAP indicated by arrow heads. Jurkats (J), CHOs (C), fyn (F1), lck (L) and ZAP (Z) transfectants were analyzed. Cell samples were paired with those from figure 4.4a.

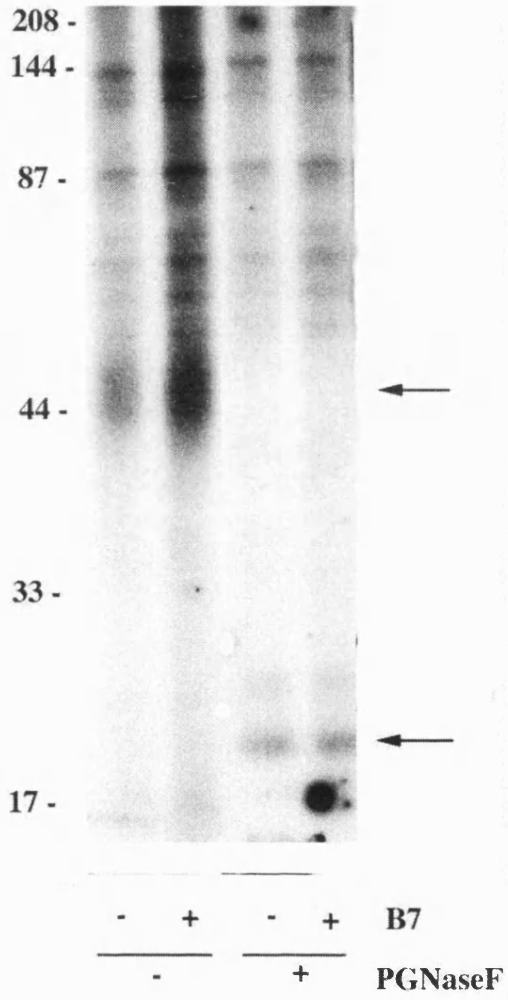


Fig. 4.5: Deglycosylation of CD28 Altered Its Electrophoretic Mobility

$1x10^7$ Lck transfectents were stimulated with $CD80^+$CHOs (B7) (+) or CHO controls (-) at a ratio of 3:1 responders to stimulators for 5 min at 37°C. CD28 immunoprecipitates were subjected to IVK reactions before treatment +/- PGNaseF, a deglycosylation agent. Products were visualized after SDS-PAGE separation by autoradiography. Upper arrow indicates position of untreated CD28, lower arrow deglycosylated CD28. A representative experiment of n=2 is shown.

4.6a Detection of p85, the Regulatory Subunit of PI 3-kinase

In order to determine the identity of the ~85 kDa protein in figure 4.4a, cellular lysates from CHO cells were used to optimize a Western blotting assay directed against p85, the regulatory subunit of PI3K (Ward et al., 1992). Figure 4.6a demonstrates that it was found possible to detect an 85 kDa protein in CHO cell lysates in addition to a number of other proteins which cross-reacted with the Western blotting antibody. However these bands migrated at different rates under SDS-PAGE and were easily distinguished from p85. Ultimately a dilution was decided upon of primary α-p85 antibody at 1/2000 and secondary antibody at 1/20000. This was an attempt to minimise non-specific contributory signals while maintaining the intensity of the p85 signal, although background signals were not ablated entirely. Thus the detection of p85 was made possible.

4.6b Effect of CD80 on Recruitment of PI 3-kinase to CD28

To investigate whether or not the 85 kDa protein observed to be phosphorylated by the ligation of CD28 by CD80 (B7) in the presence of lck or fyn (see fig. 4.4a) was p85, CD28 was immunoprecipitated from cell lines following stimulation by CD80 and the presence or absence of p85 was determined by Western blotting. Figure 4.6b showed a CD80-induced recruitment of p85 (see arrow) to Jurkats but not to a $CD28^{LOW}$ T cell line, 28N. This was consistent with a previous finding of p85 recruitment to CD28 which could be induced by CD80 in Jurkats (Ward et al., 1995). In CHO cells it was not possible to convincingly detect p85 and this may be due to assay insensitivity. Controls for CD28 expression detected more CD28 expression in transfectants than Jurkats and therefore limited CD28 was not a cause of assay insensitivity. Lck expression in transfectants however had diminished and therefore this was likely to have contributed to the difficulty of detecting p85 recruitment to CD28 should this occur in CHO transfectants.

4.7 Effect of CD28 Ligation on Acidic Sphingomyelinase Activation

A number of studies have suggested an association between CD28 ligation and ASMase activation (Boucher et al., 1995; Chan and Ochi, 1995). In order to examine whether this

may occur in CHOs, fixed $CD80^+$CHOs were used to stimulate CHOs and $CD28^+$CHOs before measurement of endogenous ASMase activity. Lysates from stimulated cells were incubated with ^{14}C sphingomyelin and the release of labelled phosphocholine due to activation of endogenous ASMase was measured by scintillation counting. It was demonstrated (fig. 4.7) that CD80-CD28 interaction could activate ASMase rapidly, facilitating a 1.7 fold increase in phosphocholine release at 3 mins, above the levels obtained where parental CHO cells were used to stimulate $CD28^+$CHOs. Activation of ASMase in $CD28^+$CHOs would indicate that CD28 can activate ASMase independent of lck/ fyn contributions. This may indicate that alternative intracellular molecules contribute to CD28-derived costimulation independent of PTKs. The low level activation of ASMase demonstrated here concurs with data from other studies where a maximal 3 fold activation of endogenous ASMase in T cell lines was seen (Edmead et al., 1996; Chan and Ochi, 1995). The slightly higher levels of activation observed in those studies may possibly be attributed to the use of T cells rather than CHOs whereby the majority of T cells which express CD28 and associated signalling effectors may have a greater capacity than transfected CHOs to interact with ASMase. Currently it is not known how CD28 activates ASMase but as ASMase is lysosomal, interaction between CD28 and ASMase would involve the translocation of one signalling entity to the other or the activation of intermediate molecules. These experiments are suggestive of a CD28-SMase interaction in CHO transfectants, but much more work needs to be performed to characterize this interaction.

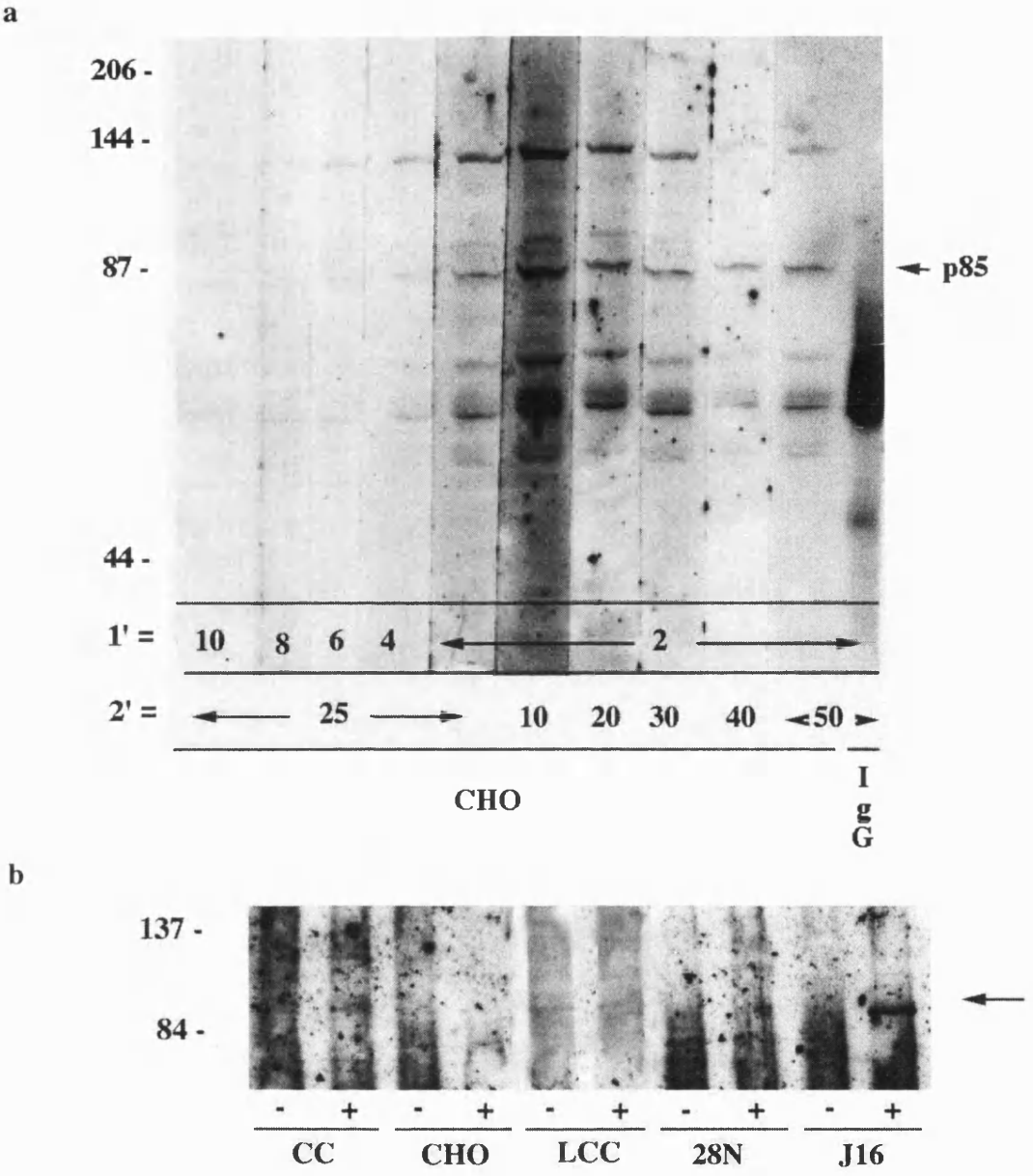


Fig. 4.6: a, Optimization of p85α Detection

1x10^6 cells/ point were lysed, their proteins separated by SDS-PAGE and p85 detection optimized by titrating α-p85 (1') and goat-α-mouse (2') antibodies against the transferred proteins. Numbers parallel to 1' and 2' relate to inverse of antibody dilution factored at 10^{-3} e.g. 2 was a 1/2000 dilution and 25 was a 1/25000 dilution.

b, Effect of CD80 on Recruitment of p85α to CD28 in Transfectants

1x10^7 cells/ lane were stimulated by CD80 (+) or CHO (-) at 37°C for 5 mins at a ratio of 3:1 responders to stimulators and CD28 was immunoprecipitated from lysates. Proteins were separated by SDS-PAGE and subjected to αp85 Western blotting. Cells lines as fig. 4.4a and 28N was a CD28^{LOW} Jurkat . A representative experiment of n=3 is shown.

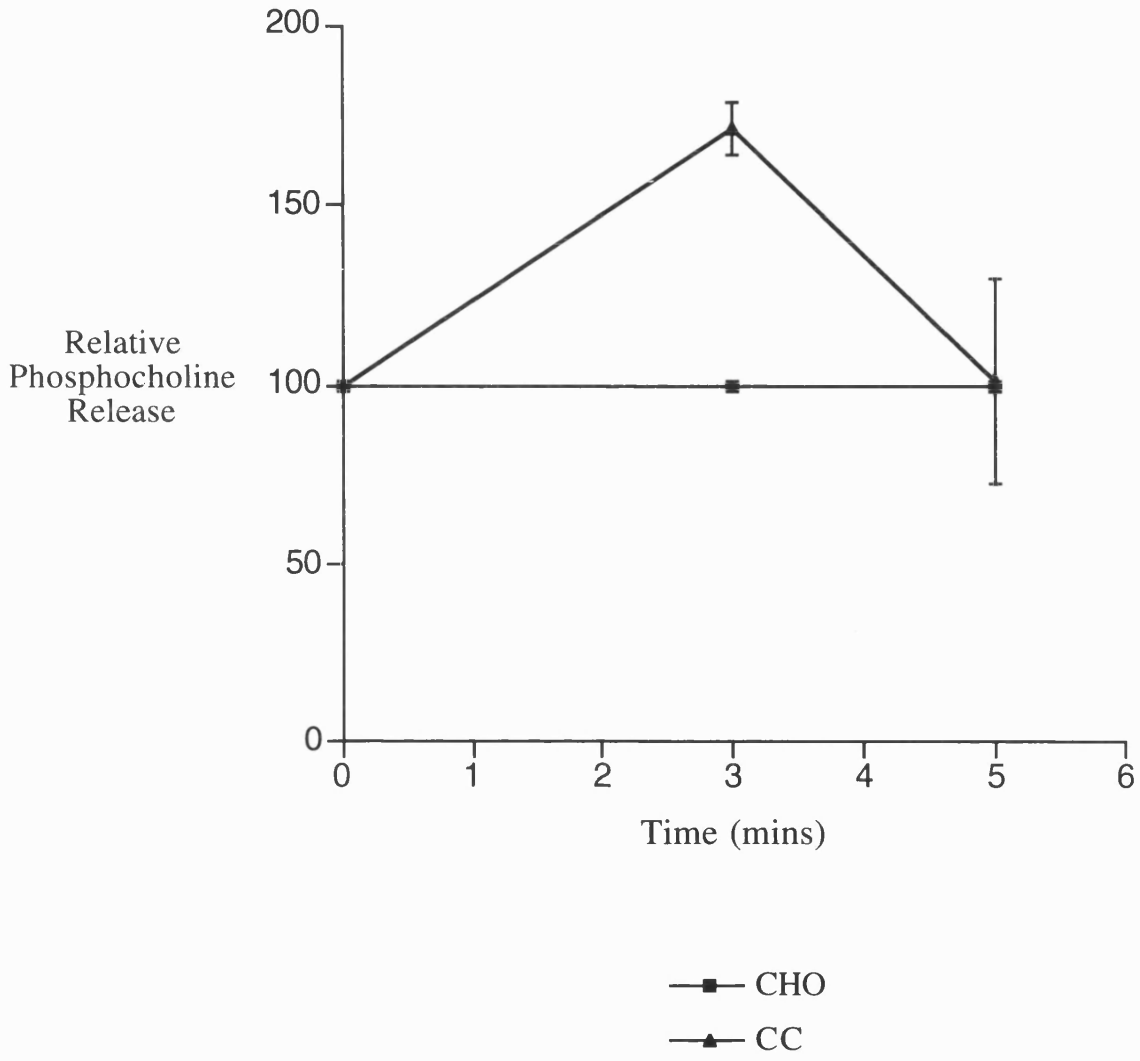


Fig. 4.7: Effect of CD28 on Acidic Sphingomyelinase Activity

ASMase activity was measured in CD80 stimulated CHOs and $CD28^{+}$CHOs (CC). $5x10^{6}$ responder cells were stimulated at 37°C by $CD80^{+}$CHOs at a ratio of 3:1 reponders to $CD80^{+}$CHOs. Samples were lysed, incubated with ^{14}C sphingomyelin and the release of labelled phosphocholine by endogenous ASMase activity was determined by scintillation. Data are presented as phosphocholine release relative to control CHO enzyme activity =100. The combined means from n=3 experiments with standard error bars are indicated.

4.8 Discussion

The experiments in this chapter attempted to address the possibility of interaction between CD28, a costimulatory receptor with no intrinsic kinase activity and the proximal signalling molecules fyn, lck, ZAP70, PI3K and ASMase. Analysis of changes in tyrosine phosphorylation, tyr/ ser/ thr phosphorylation, PI3K recruitment and ASMase activation in response to ligation of CD28 by CD80 were performed and suggested that the kinases examined made unequal contributions to CD28 signalling.

Tyrosine phosphorylation changes of CD28 immunoprecipitates assessed by Western blotting with the anti-phosphotyrosine mAb, 4G10, revealed small/ no changes in the tyrosine phosphorylation state of CD28 immunoprecipitates following stimulation by CD80. A number of possibilities may account for the low level of increase in tyrosine phosphorylation associated with CD28, despite previous reports of the importance of tyrosine phosphorylation to the costimulatory capability of CD28 (Lu et al., 1994; Vandenberghe et al., 1992). These include an incapacity of 4G10 to recognise the conformation of tyrosine phosphorylated moieties present on CD28 in CHO cells however more likely is that only small levels of tyrosine phosphorylation occur and detection of these changes were beyond the sensitivity of the assay employed. Phosphotyrosine changes in this study (fig. 4.3) and others (August and Dupont, 1994b; Hutchcroft and Bierer, 1994; Parry et al., 1997) has not been easily detected following CD28 ligation compared to phosphorylation overall (fig. 4.4a) and (Boussiotis et al., 1996; Parry et al., 1997). While CD80 did not induce increases in tyrosine phosphorylation, increases in tyr/ ser/ thr phosphorylation were observed and this suggests that the kinases were not constitutively activated as inducible changes in activation were observed in figure 4.4a for fyn and lck transfectants. For the ZAP70 transfectant no inducible changes were observed in either tyrosine or tyr/ ser/ thr phosphorylation. Therefore it is possible that the kinase was inactive or alternatively is not utilized by CD28. In order to address these points further investigation could be carried out by performing respectively Western blotting with alternative anti-

phosphotyrosine antibodies and immunoprecipitating more CD28 to overcome assay insensitivity. However the difficulty in detecting changes in tyrosine phosphorylation associated with stimulation of CD28 was addressed partly by the use of increased cell numbers in the tyrosine phosphorylation assays where 25 million cells/ lane were analyzed compared to 10 million cells/ lane when phosphorylation was measured by in vitro kinase analysis. Additionally in vitro kinase assays on directly immunoprecipitated lck/ fyn/ ZAP70 kinases to assess the degree of activation or the possibility of activation of the kinases could be performed. The necessity of tyrosine phosphorylation in contributing to costimulation has yet to be demonstrated unequivocally. Some studies have also demonstrated small tyrosine phosphorylation changes following CD28 stimulation (August and Dupont, 1994b; Lu et al., 1992), although others have recorded no tyrosine phosphorylation changes in the absence of additional stimulation from TCR/ CD3-like signals (Hutchcroft and Bierer, 1994; Hutchcroft et al., 1996; Nunes et al., 1996) or crosslinking of CD28 (Hutchcroft and Bierer, 1994; Hutchcroft et al., 1996). The results demonstrated here are consistent with the consensus arising from the data available and suggest that only limited tyrosine phosphorylation of CD28 and substrates occurs following its ligation.

The phosphorylation of CD28 immunoprecipitates following stimulation by CD80 was more marked when measured by IVK analysis than for tyrosine phosphorylation, as measured by Western blotting with the mAb 4G10. Accordingly data from IVK analysis suggests if the tyrosine phosphorylation data did indeed represent low level changes, most of the phosphorylation of CD28 immunoprecipitates occurred on ser/ thr residues. This possibility could be investigated by thin layer chromatographic analysis using phosphorylated tyr/ ser/ thr standards to compare against phosphoproteins from CD28 immunoprecipitates with and without stimulation by CD80. If ser/ thr phosphorylation of CD28 was revealed, it would imply that because lck is a tyrosine kinase, it was recruiting a ser/ thr kinase to phosphorylate CD28 rather than directly phosphorylating CD28. This would provide information on the nature of phosphorylation reactions associated with CD28 signal transduction and suggest which types of residue on the intracellular domain of CD28 may be responsible for transducing costimulatory signals. One such study

demonstrated a marked increase in tyrosine phosphorylation following antibody stimulation of CD28 (Hutchcroft et al., 1996), although data from Parry et al demonstrated comparable levels of ser and thr phosphorylation following CD80-CD28 interaction (Parry et al., 1997). This may reflect differences in the residues phosphorylated between antibody and natural ligand. The low level of inducible tyrosine phosphorylation (fig. 4.3) and the more marked level of tyr/ser/ thr phosphorylation (fig 4.4a) is in agreement with the lack of tyrosine phosphorylation of CD28 and the marked increase in ser/ thr phosphorylation following CD28-CD80 interaction observed by Parry et al (Parry et al., 1997). It is possible to suggest that differences between the levels of tyrosine phosphorylation and tyr/ ser/ thr phosphorylation may be due to ser/ thr phosphorylation. Further characterization of ser/ thr interactions with CD28 is required.

However an alternative interpretation of the tyrosine phosphorylation data presented in figure 4.3 is that the low level changes of tyrosine phosphorylation following CD28 ligation do not reflect the necessity of tyrosine phosphorylation to CD28 signal transduction. A number of observations support the suggestion that PTKs are necessary for CD28 costimulation. For example PTKs are known to be rapid signalling effectors and therefore could provide CD28 with the capability to interact rapidly with distal effectors. Furthermore mutation of the tyrosine residue in the PI3K docking site Y^{191}MNM of CD28 to phenylalanine ablates PI3K binding (Prasad et al., 1994) and IL2 production (Pages et al., 1994) where PI3K activation subsequent to CD28 ligation by CD80 has been demonstrated to be necessary for activation of human resting T cells (Ward et al., 1995; Ueda et al., 1995). Therefore this PI3K-CD28 interaction is necessary for some of the costimulatory capability of CD28 to be fulfilled and so PTKs are required to phosphorylate Y191 to facilitate PI3K binding to, and activation by, CD28. Additionally a role for lck in CD28 signalling has been suggested by the diminished production of IL2 in lck -ve Jurkats (Lu et al., 1994) under PMA, ionomycin, CD28 stimulation. However as both fyn and lck interact with the TCR (Boussiotis et al., 1996; McFarland et al., 1992) and CD28 (August and Dupont, 1994a; Raab et al., 1995; Hutchcroft and Bierer, 1994) it can be difficult to determine the contribution these PTKs make to one pathway or another. Furthermore the phosphorylation of CD28 following

ligation by CD80 (fig. 4.4a) varied depending on the cell line, suggesting that CD28 may possibly show selectivity between different PTKs in signal transduction. While lck more than fyn demonstrated a capacity to induce increases in phosphorylation of CD28 immunoprecipitates, ZAP70 transfectants did not. This may be due to differential recruitment and/ or activation of these PTKs by CD28. Alternatively it may be the case that ZAP70, although detectable by Western blotting was not expressed as a kinase which could be activated. A further control to assess this possibility may be performed by IVK analysis of immunoprecipitated PTKs which would reveal if all three kinases could be activated. Interestingly similar experiments performed by Raab (Raab et al., 1995) also found that despite expression of ZAP70, which in an IVK assay was found to be active, ZAP70 transfectants did not stimulate CD28 phosphorylation while lck or fyn could (Raab et al., 1995). One reason why this may occur may reside in the lack of an ITAM sequence on the intracellular domain of CD28 which has been shown to be necessary for ZAP70 activation following TCR ligation (Hatada et al., 1995). Thus lck is the best candidate to date for transducing CD28 signals.

Following ligation of CD28 by CD80 in the fyn and lck transfectants increased phosphorylation of three bands was observed. These migrated under reducing conditions as proteins of molecular weights 44, 85 and 110 kDa which coincide with the molecular weights of CD28 (Aruffo and Seed, 1987; Hutchcroft et al., 1996; Parry et al., 1997) and the regulatory and catalytic subunits of PI3K respectively (Ward et al., 1992). This is highly consistent with the possibility that ligation of CD28 by CD80 leads to increased phosphorylation of proteins demonstrated to be CD28 and PI3K in other studies (Lu et al., 1994; August and Dupont, 1994b; Parry et al., 1997). Strong support for the possibility that the broad 44 kDa band was CD28 is derived from deglycosylation data (fig. 4.5) where deglycosylation of CD28 immunoprecipitates resulted in an altered electrophoretic mobility of only one band from 44 kDa to 23 kDa. The breadth of this band may be accounted for by considerable glycosylation of CD28 on 5 glycosylation sites on its extracellular domain (Aruffo and Seed, 1987). This shift in molecular weight demonstrated in this study following deglycosylation concurs with nucleotide sequence data which predicts CD28 to exist as a 23 kDa molecule (Aruffo and Seed, 1987) and is

in agreement with another study (Hutchcroft et al., 1996) demonstrating a similar altered electrophoretic mobility of a band present in CD28 immunoprecipitates. This data would support the possibility that the broad 44 kDa band observed in figure 4.4 was indeed CD28.

The relationship between CD28 ligation by CD80 and PI3K activation may be investigated by analysis of D3 phosphoinositol lipid production following CD80 stimulation of CD28 as demonstrated by Ward et al (Ueda et al., 1995). The experiments in figure 4.6b demonstrated that following CD80 stimulation of CD28 in Jurkats, there was an inducible recruitment of p85 to CD28 in agreement with data from other reports indicating CD28 ligation leads to the recruitment of PI3K (Ward et al., 1995; Stein et al., 1994; Ueda et al., 1995; Prasad et al., 1994; Pages et al., 1994; Ward et al., 1993; Cai et al., 1995; Pages et al., 1996). This would suggest that the inducibly phosphorylated bands migrating at 85 and 110 kDa observed in figure 4.4b may have been PI3K, although additional support for this hypothesis may be supplied by Western blotting against the p110 subunit of PI3K. In CHO transfectants attempts to identify the smaller molecule as the regulatory subunit of PI3K by immunoblotting were hampered for a number of reasons, although the primary cause was likely to be the transient expression of PTK gene product. Over a number of passages it became evident that the phosphorylation response of the lck transfectants following CD80-CD28 interaction decreased. This may be seen when comparing the phosphorylation response of early transfectants (figure 4.5, lanes 1 and 2) with that of highly passaged transfectants (lanes 3 and 4). The phosphorylation experiments represented in figures 4.3 and 4.4 utilized early lck transfectants, which when Western blotted had demonstrable lck expression, whereas the later passaged lck populations (figure 4.5 lanes 3 and 4 and 4.6b) had undetectable lck expression. Therefore the usefulness of a mixed population of transfectants was limited by time. Possible reasons for the decrease in PTK expression over time include a greater rate of proliferation of non transfected cells or transfected cells not expressing the PTK. Another problem concerning the specific detection of p85 was the relatively high degree of cross-reactivity between the primary anti-p85 antibody (JS14) and other proteins present in both CHO cellular lysates (figure 4.6a) and CD28

immunoprecipitates (figure 4.6b). It would be interesting to repeat these experiments with fresh transfectants and different blotting antibodies against p85.

The activation of ASMase following ligation of CD28 by CD80 demonstrated in figure 4.7 is in agreement with previous data (Boucher et al., 1995; Edmead et al., 1996; Chan and Ochi, 1995) indicating a possible role for ASMase in CD28 costimulation. However the functional relevance of ASMase activity in resting human T cells has yet to be determined. Although there is little understanding of the mechanism by which CD28 and ASMase interact, a number of observations suggest areas of future research which may provide information on the identity/ mechanism of effectors participating in the interaction of CD28 and ASMase. Accordingly ASMase is known to be localized to lysosomes (Spence, 1993) and recent data indicates that the level of expression of CD28 on the cell surface is in part regulated by degradation in lysosomes (Cefai et al., 1998). Thus an opportunity for CD28 and ASMase interaction is provided when CD28 translocates to lysosomes. If lysosomes represent the location of interaction, the question of how ASMase interacts with CD28 still remains. Currently there is no data on this subject but one may suggest an interaction between CD28, ASMase and one or more intracellular effectors interacting with the tyrosine residue Y191. This suggestion arises from a CD28 cytoplasmic mutant Y191F which showed persistent surface expression with markedly reduced intracellular localisation of CD28 (Cefai et al., 1998). Therefore molecules interacting with Y191 facilitate the translocation of CD28 from the membrane to lysosomes. A number of candidate molecules exist which can bind the phosphorylated Y191 residue including p72 itk, which is reported to negatively regulate CD28/ T cell function (Liao et al., 1997); PI3K which is proposed to be necessary for costimulation of resting human T cell activation ie IL2 production (Ward et al., 1995; Ueda et al., 1995; Ward et al., 1993) and the adapter protein GRB2 (Schneider et al., 1995a), which may facilitate interactions between CD28 and the small G proteins Rac1 and Cdc42 (Kaga et al., 1998a) ultimately leading to activation of kinase cascades eg involving PAK1 and MEKK1 (Kaga et al., 1998a) and so the activation of the AP-1 transcriptional promoter site of the IL2 gene. Of these possible effectors which may/ may not interact in CD28-

ASMase signalling, preliminary data are available on an interaction between CD28 and PI3K in the regulation of CD28 surface expression. Accordingly a significant proportion (one third) of the lysosomally-targeted internalized CD28 associates with PI3K (Cefai et al., 1998).

In conclusion we demonstrated that lck transfectants markedly phosphorylated molecules likely to be CD28 and corresponding with the molecular weight of PI3K subunits in response to CD80-CD28 interaction. Deglycosylation of CD28 immunoprecipitates resulted in a specific alteration in the molecular weight of only one molecule from a broad 44 kDa band to a more defined band of 23 kDa, consistent with the migration of CD28 before and after deglycosylation (Hutchcroft et al., 1996). Fyn transfectants demonstrated a similar phosphorylation of CD28 substrates, although to a less intense degree than lck, despite higher levels of expression of fyn than lck relative to the levels of these kinases in Jurkats. This suggests that CD28, at least in our CHO transfectant model, may utilize lck in preference to fyn in CD28 transduction pathways. By contrast ZAP70 appeared to contribute nothing to CD28 phosphorylation and it may be suggested that this is either because it was not utilized by CD28 or that it was expressed as a defective kinase. We also demonstrated the recruitment of p85, the regulatory subunit of PI3K to CD28 following CD28 ligation by CD80 in Jurkats and the activation of ASMase following CD80-CD28 interaction in $CD28^{+}$CHOs. The data presented here represent new findings in that an inducible contribution to CD28 signalling due to the presence of lck or fyn, without contribution from other T cell specific effectors, has not previously been demonstrated and that activation of ASMase by CD28 independent of fyn/ lck suggests that alternative, PTK-independent CD28 signalling pathways exist.

Chapter 5

The Effect of Sphingomyelinase or C2 Ceramide on Resting T cell Proliferation, Viability and Surface Marker Expression

Resting T cells are known to require two signals to mount an effective immune response. The previous chapter attempted to define some of the proximal signalling elements downstream of CD28 involved in transducing the costimulatory effects of CD28. One such element, acidic sphingomyelinase(ASMase), was suggested to be responsible for the costimulatory effects of CD28 (Boucher et al., 1995; Edmead et al., 1996; Chan and Ochi, 1995), although it was evident from proliferative levels that even at an optimal dose, ASMase did not show a quantitatively similar response to CD28 stimulation. This suggests that the costimulatory function of CD28 may be facilitated by ASMase and additional signals. This chapter attempts to analyze the effects of sphingomyelinase activity on resting T cells by examining the proliferation, activation and viability of costimulated T cells. All viability data are paired with the proliferation figures preceding them.

5.1 Assessment of Acidic and Neutral Sphingomyelinase Activity in *Staphylococcus aureus* Sphingomyelinase

In order to define whether the activity of SMase, derived from Staphylococcus aureus, had a neutral or an acidic pH optimum, as at least two SMases exist with different pH requirements (Spence, 1993), SMase was assessed for its capability to release phosphocholine from sphingomyelin under acidic or neutral reaction conditions. Dilutions of SMase were incubated with ^{14}C sphingomyelin at 37°C for 2 hours and the release of radiolabelled phosphocholine was measured by scintillation. Figure 5.1 demonstrates that SMase comprises both acidic and neutral SMase activities which released comparable levels of phosphocholine.

5.2 The Ability of Sphingomyelinase, C2 Ceramide or Phosphocholine to Costimulate the Proliferation of Resting T cells

To determine whether SMase or its products were capable of replacing CD28-derived signals as a costimulus for resting T cell proliferation, T cells were stimulated by αCD3 mAb and fixed $CD80^+$CHOs , SMase, C2 ceramide or phosphocholine. Proliferation was measured by assessing the levels of ^3H thymidine uptake.

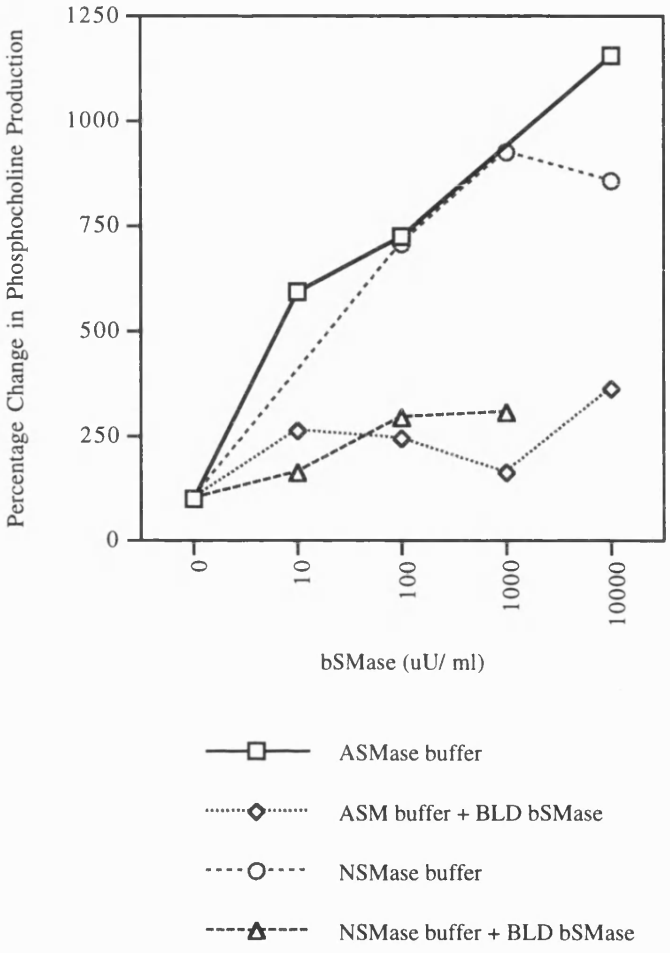


Fig. 5.1: Neutral and Acidic Sphingomyelinase Activities of Staphylococcus aureus Sphingomyelinase

Neutral and acidic SMase activity in S. aureus SMase was assessed. Titrations of SMase were incubated in neutral or acidic reaction buffers with ^{14}C sphingomyelin. The release of radiolabelled phosphocholine was subsequently measured. The effect of denaturation of the enzyme preparation on SMase activity was assessed in a parallel assay with boiled (BLD) SMase. Data are derived from a single determination at one sample/ dilution.

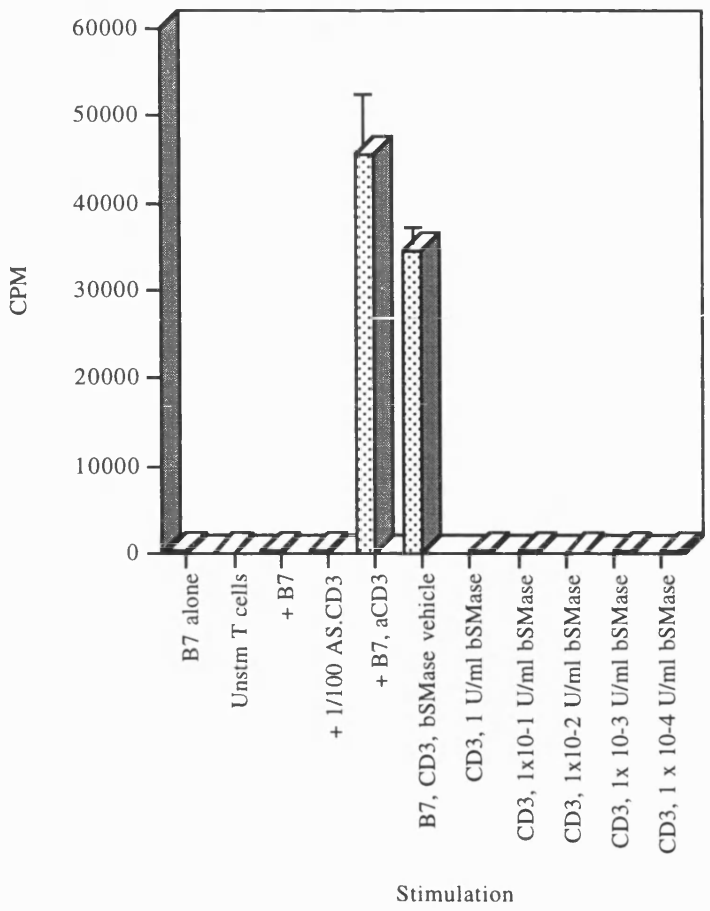


Fig. 5.2a: The Ability of CD80 or Sphingomyelinase to Costimulate Resting T cell Proliferation

Resting T cells were left unstimulated, or incubated with αCD3 (1/ 100 dilution of ascites) alone or in the presence of fixed CD80 cells or increasing concentrations of SMase for 72 hours. bSMase vehicle was present at a concentration equivalent to 1U/ ml bSMase. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SD indicated by horizontal bars).

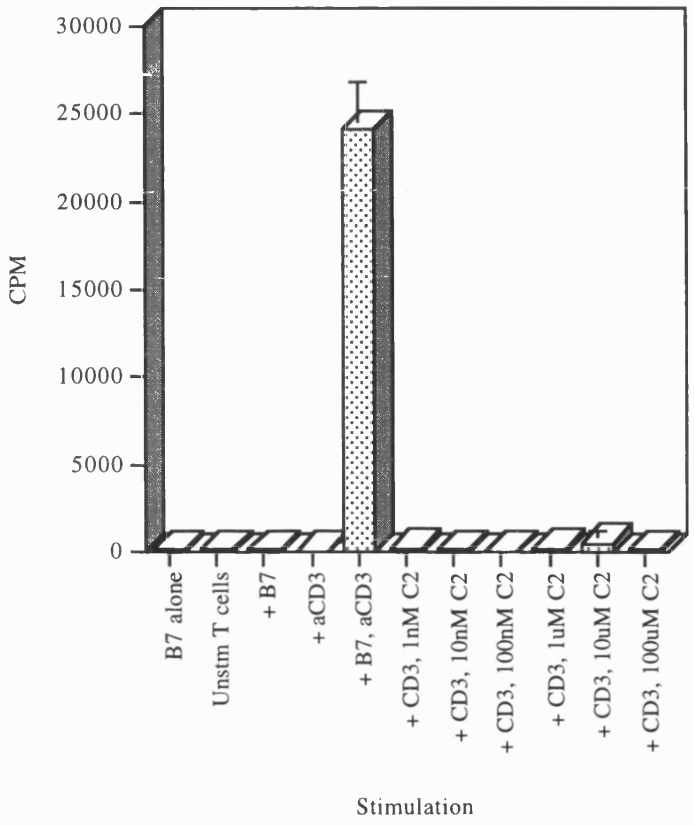


Fig. 5.2b: The Ability of CD80 or C2 Ceramide to Costimulate Resting T cell Proliferation

Resting T cells were left unstimulated or incubated with αCD3 mAb (1/ 100 dilution of ascites) alone or in the presence of fixed CD80 cells or increasing concentrations of C2 ceramide for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SD indicated by horizontal bars).

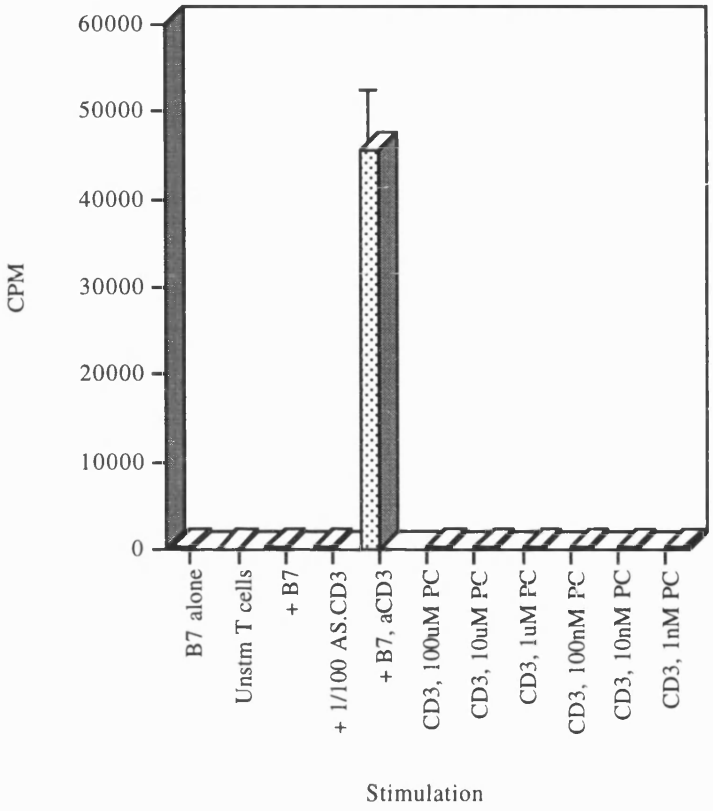


Fig. 5.2c: The Ability of CD80 or Phosphocholine to Costimulate Resting T cell Proliferation

Resting T cells were left unstimulated, or incubated with αCD3 (1/ 100 dilution of ascites) alone or in the presence of fixed CD80 cells or increasing concentrations of phosphocholine (PC) for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SD indicated by horizontal bars).

While CD80 and αCD3 costimulation of resting T cells induced a significant proliferative response, neither a range of SMase (fig. 5.2a), nor C2 ceramide (fig. 5.2b) nor phosphocholine (fig. 5.2c) titrations could costimulate resting T cell proliferation. This is in contrast to previous suggestions that a SMase activity was sufficient to induce at least some of the proliferative response which CD28 ligation caused (Boucher et al., 1995; Chan and Ochi, 1995). Therefore CD80 but not SMase, C2 ceramide nor phosphocholine was capable of costimulating resting T cells under the conditions employed in this study.

5.3 Effect of Sphingomyelinase or C2 Ceramide on Unstimulated Resting T cell Viability

In order to assess whether the inability of SMase or C2 ceramide to costimulate T cell proliferation was due to their toxicity i.e. a capacity to cause cell death, the viability of unstimulated resting T cells was assessed following incubation in medium, SMase or C2 ceramide using a propidium iodide (PI) exclusion assay. Cells which have intact membranes prevent PI from entering the cell and therefore how low/ -ve fluorescence compared to cells with damaged membranes ie dead/ dying cells. Both SMase at 7.5 x 10^{-5} U/ ml and C2 ceramide (fig. 5.3) decreased the viability of resting T cells. At 50μM C2 ceramide, 63.1% of the cells were positive for FL2 compared to 4.63% of unstimulated T cells, although some of this effect was attributable to its vehicle ethanol which caused 25.3% of the population to lose viability. SMase induced 22.4 % of the population to lose viability. SMase and C2 ceramide reduce unstimulated resting T cell viability and this concurs with other reports where C2 ceramide reduced cell viability, possibly through apoptosis (Wolff et al., 1994; Gulbins et al., 1995; Jarvis et al., 1994).

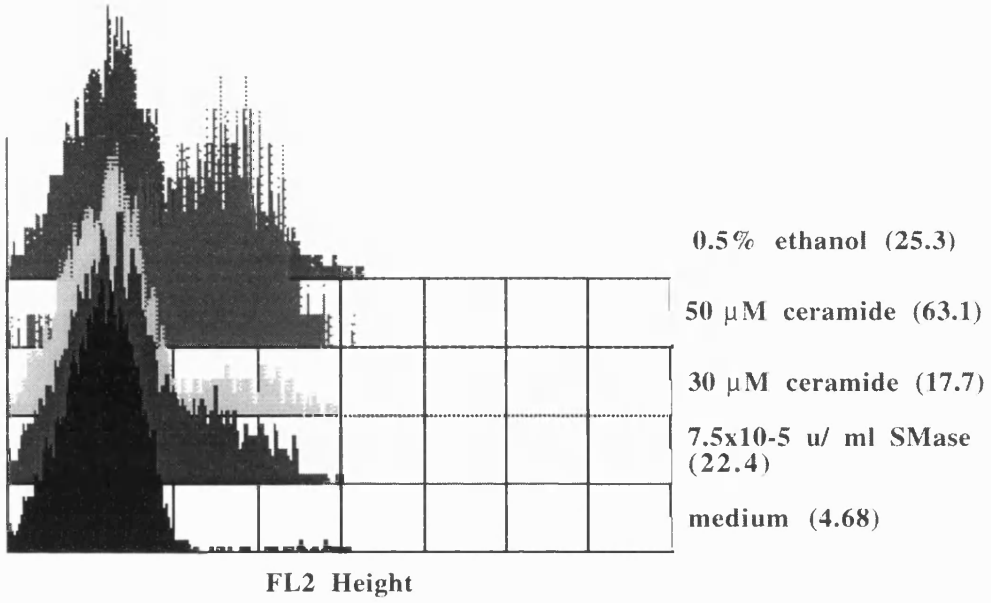


Fig. 5.3: Effect of Sphingomyelinase/ C2 Ceramide on Viability of Unstimulated T cells

Resting T cells (2 x 10^5/ point) were left unstimulated (medium) or incubated with ceramide, its vehicle (ethanol) or SMase and analyzed by FACS for viability. Propidium iodide (PI, 10µg/ ml, FL2) uptake which is proportional to cell mortality was used to assess the effect of treatments on viability. Figures in brackets indicate the percent of cells showing mortality relative to the control where 4.68% of the cells did not exclude PI. A representative experiment of n=3 is shown.

5.4 Ability of Sphingomyelinase or C2 Ceramide to Modulate Costimulated Resting T cell Proliferation and Viability

To assess the effect of SMase and its products on T cells receiving costimulation, αCD3, CD80 stimulated cultures of resting T cells were incubated with/ without titrations of SMase, C2 ceramide and phosphocholine for 72 hours. Proliferation was measured by ^{3}H thymidine incorporation and from duplicate cultures viability was assessed by PI exclusion using FACS analysis. It was found that SMase from $5x10^{-5}$ to $5x10^{-3}$ U/ ml decreased or ablated the proliferation of costimulated resting T cells (fig. 5.4a) independent of any effect of the vehicle. The vehicle was added to culture at a dilution corresponding to the highest titre of SMase. At concentrations of $1x10^{-5}$ U/ ml SMase and lower, little or no decrease in proliferation of costimulated resting T cell cultures was observed relative to the vehicle control. In summary concentrations of SMase in excess of 5 $x10^{-5}$ U/ ml decreased proliferation.

Interestingly the viability data (fig. 5.4b) showed that at concentrations of SMase at $5x10^{-5}$ U/ ml there was no decrease in viability. Thus the decrease in proliferation observed at $5x10^{-5}$ U/ ml was not caused by death of the culture overall. Between $5x10^{-5}$ U/ ml and $1x10^{-4}$ U/ ml no or little increase in cell death was observed (data not shown). At $1x10^{-3}$ U/ ml and above cell death increased up to 56.6% from 16.2% where SMase was not added to the culture. However for costimulated T cells SMase could, at certain concentrations, decrease proliferation without increasing cell death.

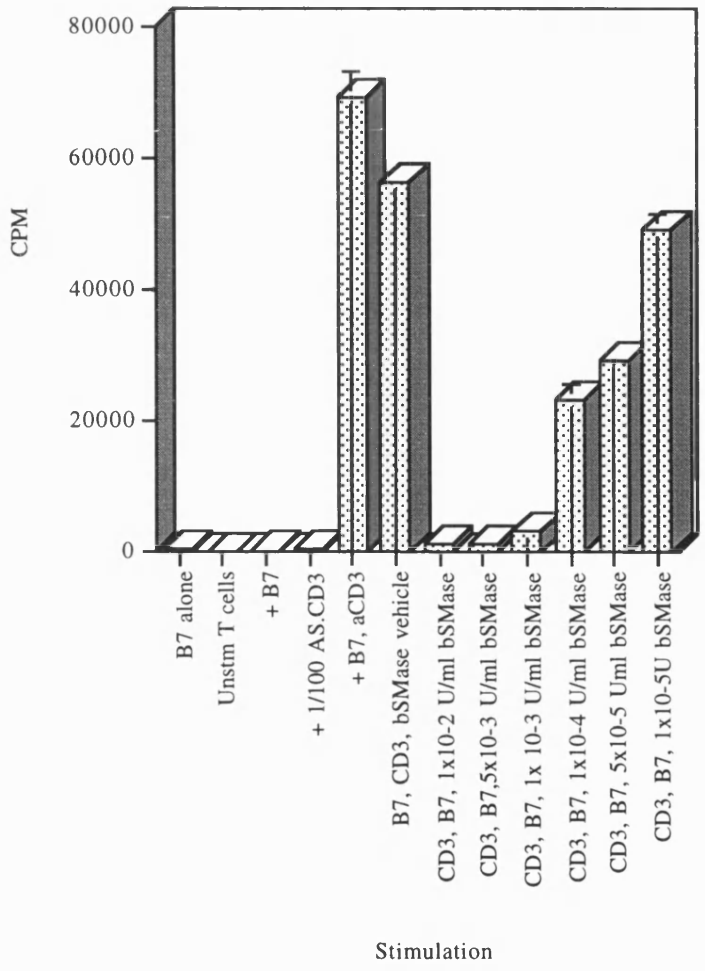


Fig. 5.4a: The Effect of Sphingomyelinase on the Proliferation of Resting T cells Stimulated by αCD3, CD80

Resting T cells were left unstimulated, or incubated with αCD3 (1/ 100 dilution of ascites) in the presence of fixed CD80 cells with or without (bSMase vehicle at a concentration equivalent to $1x10^{-2}$ U/ ml bSMase) increasing concentrations of bSMase for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SD indicated by horizontal bars).

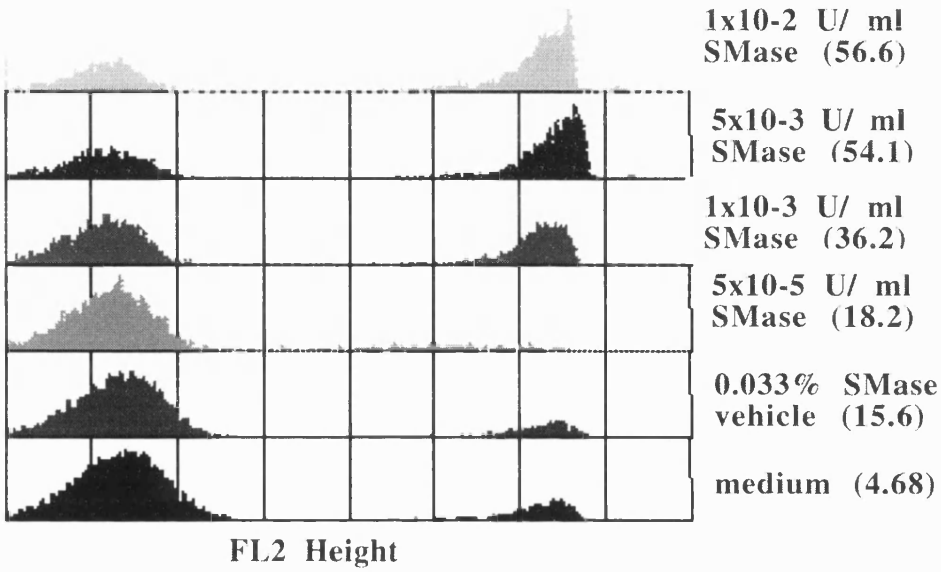


Fig. 5.4b: Effect of Sphingomyelinase on the Viability of Costimulated T cells

αCD3, B7 stimulated resting T cells (2 x 10^5/ point) were incubated for 72 hours with/ without SMase and analyzed by FACS for viability. Propidium iodide (PI, 10μg/ ml, FL2) uptake by the cells, which is proportional to cell mortality, was measured by FACS. Figures in brackets refer to the percent of cells showing positive fluorecence i.e. beyond the level recorded in the viable portion of the costimulated T cell sample. These data are paired with the data in figure 5.4a.

C2 ceramide also caused a dose dependent inhibition of proliferation (fig. 5.4c) where costimulated T cell proliferation was ablated by the addition of 100μM C2 ceramide. Costimulated cell viability was resistant to the effects of C2 ceramide (fig. 5.4d) and only at 100μM C2 ceramide, the highest concentration employed in this assay (and the one which ablated proliferation) was a marginal reduction in viability observed. While costimulated T cells had 20.5% death, the samples with the addition of 100μM C2 ceramide had 24.3% death. C2 ceramide, like SMase, could reduce proliferation without promoting a loss in viability of the costimulated T cell population.

5.4e Ability of Phosphocholine to Modulate Costimulated T cell Proliferation

An analysis of the capability of the other metabolite of sphingomyelin, namely phosphocholine, to alter costimulated T cell proliferation was made by a ^{3}H thymidine incorporation assay. Following 72 hours of costimulated culture, phosphocholine could be seen to have had negligible effect on costimulated T cell proliferation. Figure 5.4e illustrates the lack of effect of the highest concentration of phosphocholine, which was representative of a titration from 10-100μM phosphocholine. Phosphocholine, unlike SMase and C2 ceramide, could not inhibit costimulated T cell proliferation.

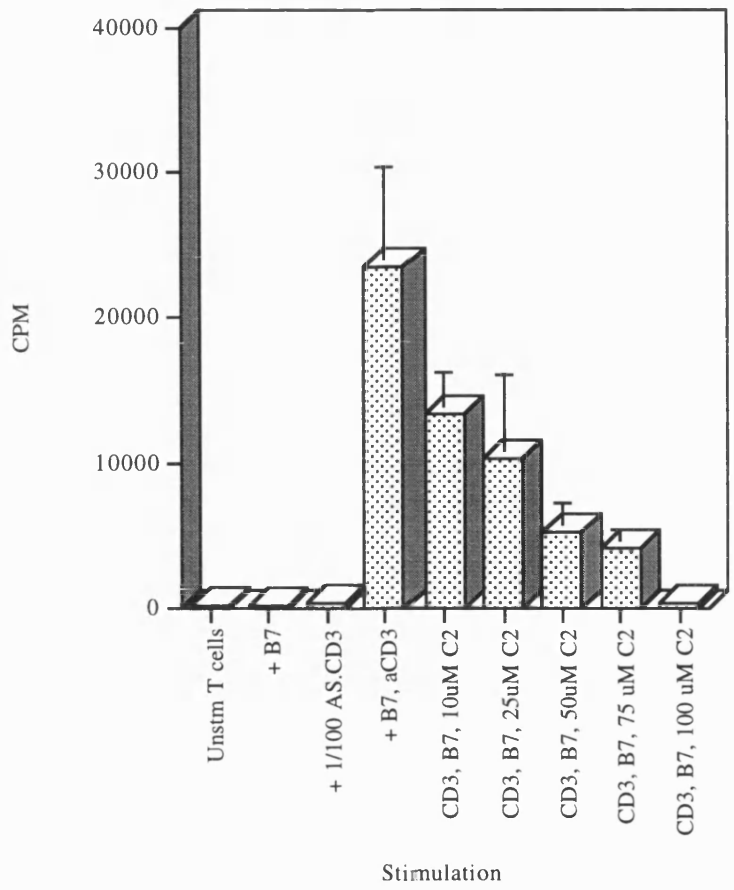


Fig. 5.4c: The Effect of C2 Ceramide on the Proliferation of Resting T cells Stimulated by αCD3, CD80

Resting T cells were left unstimulated, or incubated with αCD3 (1/ 100 dilution of ascites) in the presence of fixed CD80 cells with or without increasing concentrations of C2 ceramide for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SD indicated by horizontal bars).

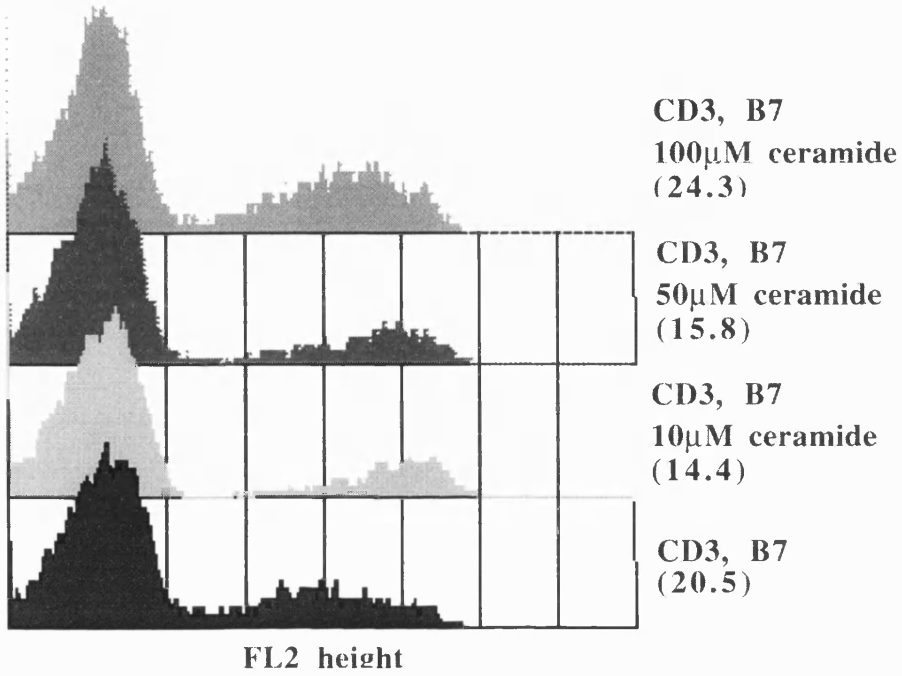


Fig. 5.4d: Effect of Ceramide on the Viability of Costimulated T cells

αCD3, CD80 stimulated resting T cells (2 x 10^5/ treatment) were incubated for 72 hours with/ without ceramide and analyzed by FACS for viability. Propidium iodide (PI, 10μg/ ml, FL2) uptake, proportional to cell mortality, was measured by FACS. Figures in brackets indicate the percent of cells with FL2 levels greater than costimulated T cells. These data are paired with figure 5.4c.

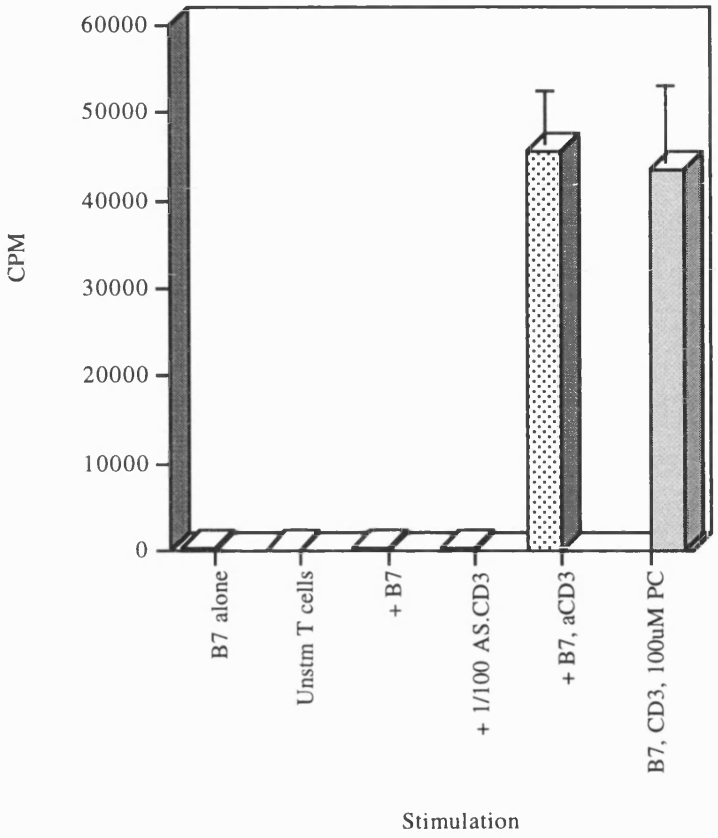


Fig. 5.4e: The Effect of Phosphocholine on the Proliferation of Resting T cells Stimulated by αCD3, CD80

Resting T cells were left unstimulated or incubated with αCD3 (1/ 100 dilution of ascites) in the presence of fixed CD80 cells with or without increasing concentrations of phosphocholine (PC) for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SD indicated by horizontal bars). The highest dose of PC was representative of its effect at any dose from 10-100μM.

5.5 Acidic Denaturation of SMase

In order to check that the effect of the SMase preparation supplied was based on enzymatic activity, its physical properties and ability to limit proliferation were assayed before and after denaturation. Denaturation was performed by incubation of an aliquot of SMase for 10 minutes in concentrated hydrochloric acid, and subsequent neutralization with sodium hydroxide. Aliquots were reserved for analysis of physical properties by SDS-PAGE (fig. 5.5a) or by their effects on proliferation of costimulated T cells (fig. 5.5b).

In the untreated lane, i.e. no acid denaturation, two bands were visible at ~40 and 32 kDa (see lower arrows, fig. 5.5a), possibly representing entities within the SMase preparation responsible for its acidic and neutral activity. The Swiss protein bank predicts a molecular weight of 37.3 kDa for SMase from its amino acid sequence although in a review of SMases from different species widely varying molecular weights were reported from 23 and 34 kDa for bacterial SMase to 70-120 kDa for human SMases (Spence, 1993). The reason for the wide variation in molecular weights of SMases was unknown although glycosylation was suggested as a mechanism contributing to the existence of proteins migrating at different rates (Spence, 1993) and S. aureus SMase has two glycosylation sites (Swiss protein data bank). The appearance of a single protein band following denaturation concurs with the suggestion that SMase readily forms aggregates (Spence, 1993). An early report detailing the purification of β haemolysin (Wadstrom and Mollby, 1971), found to be composed of SMase activity, showed the presence of molecules of 33 and 38 kDa which is comparable to that illustrated for SMase (fig. 5.5a, lane 1).

A site sensitive to acid cleaving was found (Swiss Protein data bank) carboxyl to proline residue 261 in the mainly polar but uncharged sequence HNSTWDP^{261}QSNSI. This may create an opportunity for electrostatic interactions to form an agglutinate. Therefore the single higher molecular weight product may represent an agglutinate of the other two species. Subsequent analysis of the ability of denatured SMase to modulate proliferation (fig. 5.5b) showed that the denatured SMase had little effect on proliferation beyond that

of the SMase vehicle. However the same concentration of native, i.e. non-denatured, SMase ablated proliferation. It would appear then that a component of the SMase preparation sensitive to acidic denaturation was responsible for decreased proliferation of resting T cells and this was likely to be its enzymatic activity.

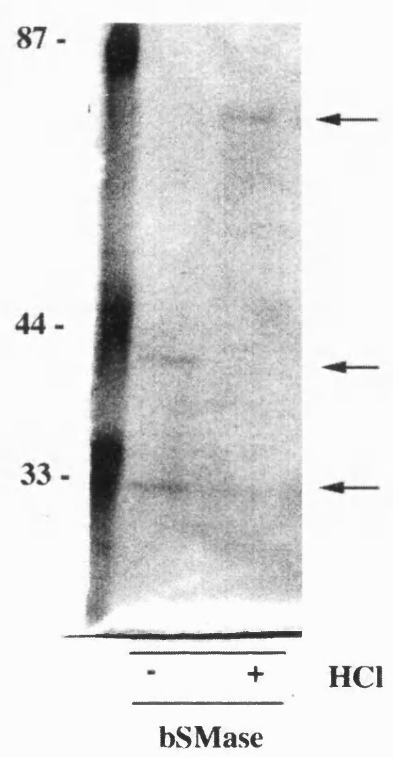


Fig. 5.5a: Denaturation of Sphingomyelinase by Concentrated Hydrochloric Acid

Equal aliquots of bSMase were denaturated (+) or not (-) by a 10 minute incubation with concentrated HCl followed by neutralization with NaOH. 1µg was analyzed by SDS-PAGE and and the products were visualized by immersion of the gel in Coomassie Blue protein stain. Lower arrows indicate positions of non-denatured bSMase and upper arrow position of single denatured product. A single determination was performed.

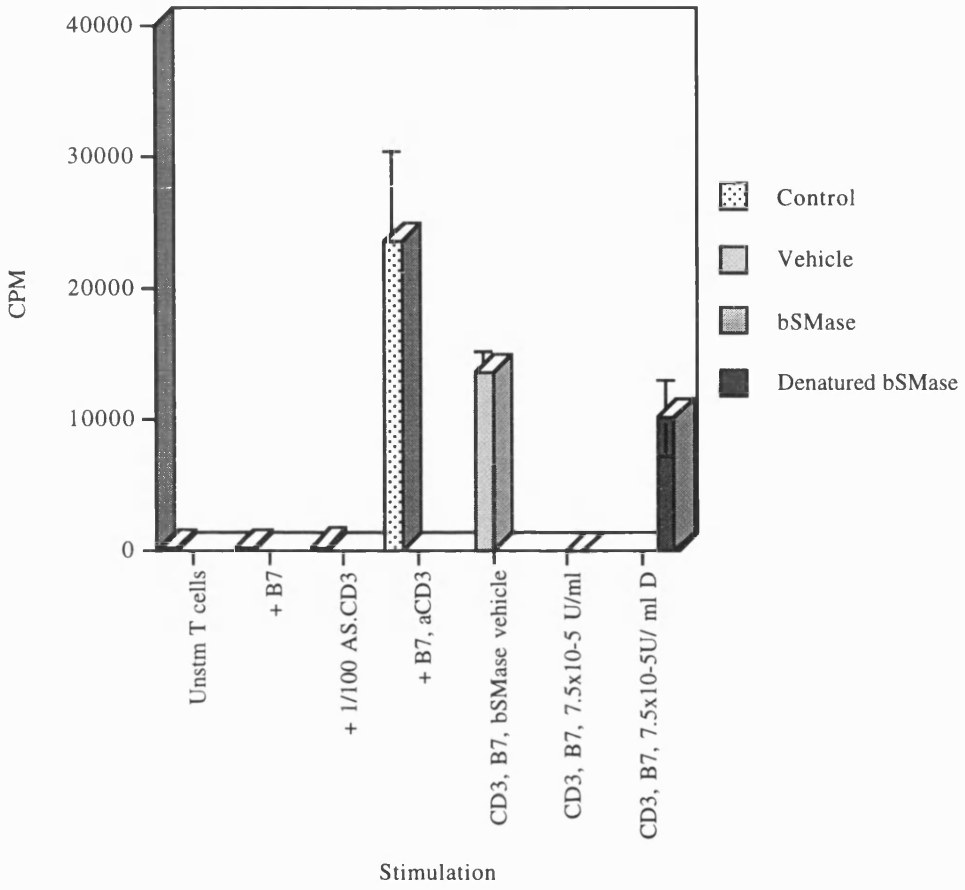


Fig. 5.5b: Effect of Denatured Sphingomyelinase on the Proliferation of Resting T cells Stimulated by αCD3, CD80

Resting T cells were left unstimulated or incubated with αCD3 (1/ 100 dilution of ascites) in the presence of fixed CD80 cells, or with SMase vehicle at a concentration equivalent to 7.5 x 10^{-5} U/ ml, SMase or denatured (D)SMase for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single experiment (SD indicated by horizontal bars).

5.6 Ability of Sphingomyelinase or C2 Ceramide to Alter the Viability and Proliferation of Blasts and Jurkats

To determine whether SMase could inhibit the proliferation of T cells with different proliferative requirements, ^{3}H thymidine incorporation assays were performed following the incubation of T cell blasts and Jurkats with/ without SMase for 24 hours. The effect of C2 ceramide on Jurkat proliferation and viability was also examined following the incubation of Jurkats in C2 ceramide over 24 hours to determine whether SMase and C2 ceramide had similar effects. Preliminary data (fig. 5.6a) illustrates that SMase did not reduce the proliferation, nor viability (fig. 5.6c), of T cell blasts and figure 5.6b shows the same was observed for Jurkats. As SMase could not alter the proliferation nor the viability of T cells with minimal proliferation requirements even at a concentration which caused 56.6% death of costimulated T cells (fig. 5.4b), it would appear that SMase has a stage specific effect.

Of interest however was the effect of C2 ceramide on the proliferation (fig. 5.7a) and viability (fig. 5.7b) of Jurkats. These preliminary data indicate that C2 ceramide effected a dose dependent proportional increase in mortality and loss of proliferation. Although the mechanism by which the Jurkats died was not analyzed, it may be due to apoptosis as ceramide has been reported to induce apoptosis (Verheij et al., 1996; Gulbins et al., 1995; Cifone et al., 1993; Higuchi et al., 1996). At 75μM C2 ceramide, there is 98% mortality of Jurkats (fig. 5.7b) and a similar decrease in proliferation could be observed (fig. 5.7a). While the proliferation of costimulated T cells was inhibited in the presence of C2 ceramide (fig. 5.4c), their viability was affected very little (fig. 5.4d), unlike Jurkats (fig. 5.7b) or unstimulated T cells (fig. 5.3). Therefore C2 ceramide appeared to induce two types of effect. The first which could be seen in costimulated resting T cells was that of inhibiting proliferation. The second effect which was the reduction of cell viability was only manifest in T cells not receiving primary or costimulatory signals i.e. Jurkats and unstimulated resting T cells. Therefore it would appear that C2 ceramide may induce an inhibition of proliferation and in the absence of costimulation induce cell death.

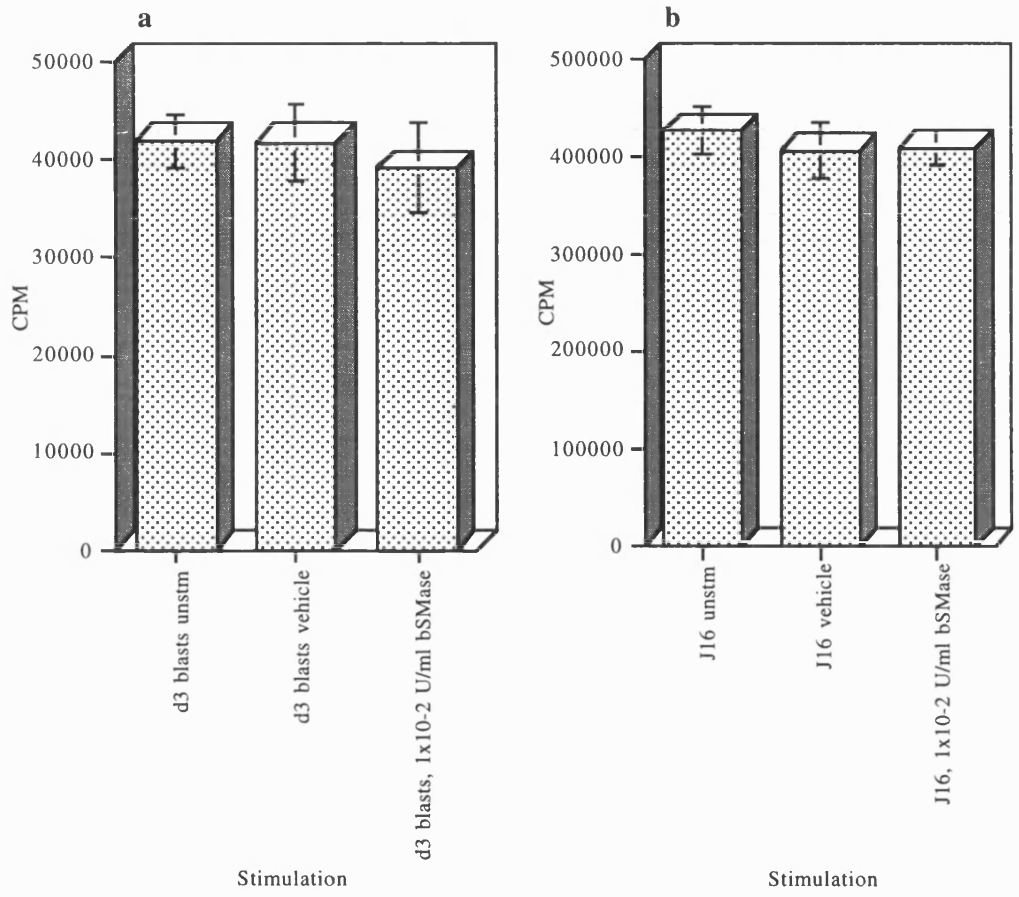


Fig. 5.6a+b: Effect of SMase on the Proliferation of T cells Blasts and Jurkats

a) T cell blasts or b) Jurkats were incubated with/ without increasing concentrations of bSMase for 24 hours. 0.03% v/v bSMase vehicle was included at a concentration equivalent to $1x10^{-2}$ U/ ml bSMase. The highest dose of bSMase used in the assay is shown as a representative example. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single experiment (SD indicated by horizontal bars).

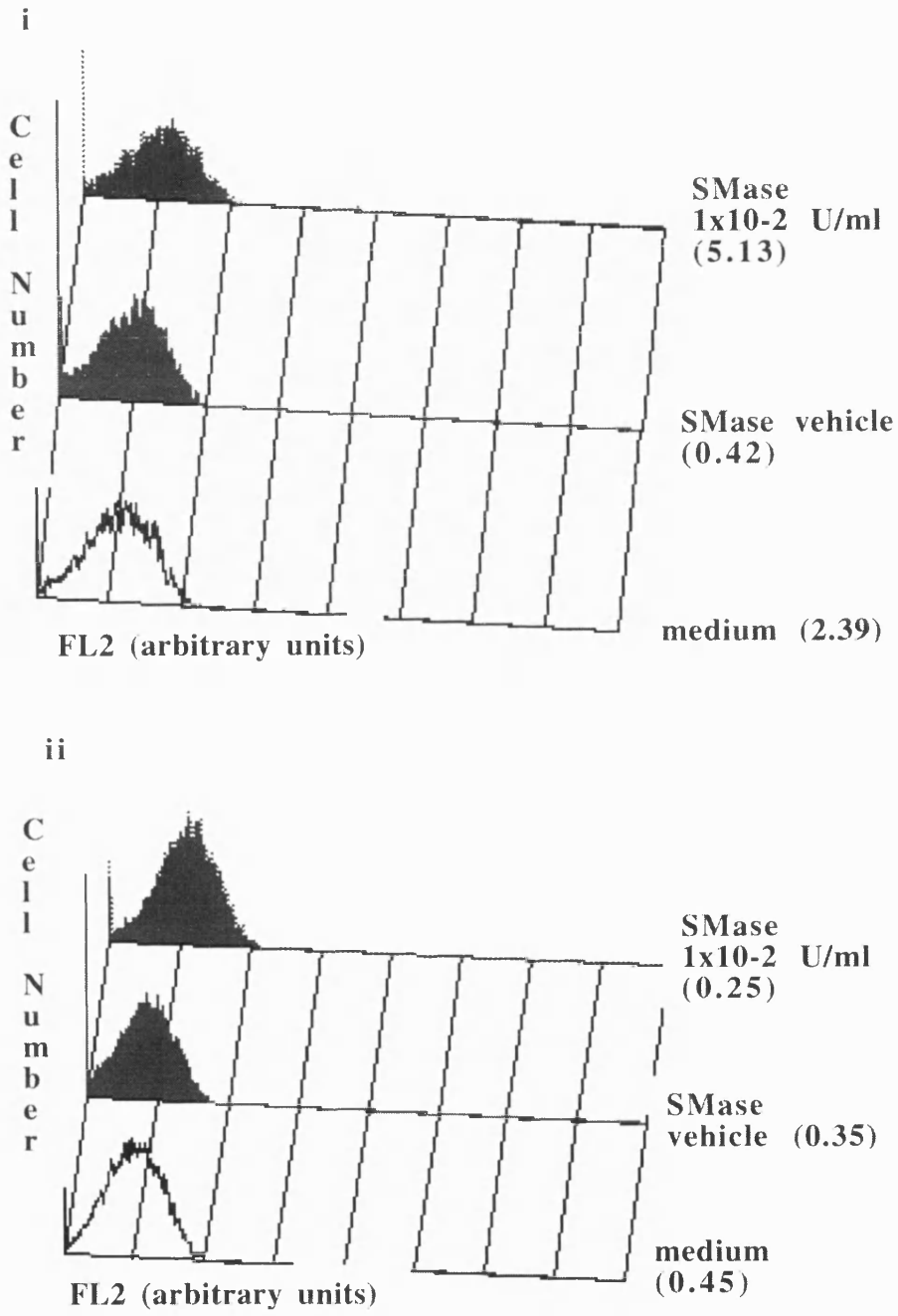


Fig. 5.6c: Effect of SMase on the Viability of T cell Blasts and Jurkats

T cell viability +/- SMase was assessed by a PI exclusion assay. $2x10^5$ i) blasts or ii) Jurkats/ point were incubated with/ without SMase for 24 hrs. PI (10µg/ ml) uptake, proportional to cell mortality, was measured as FL2. Figures in brackets indicate the percent of cells recorded with greater FL2 than the control. These data are paired with those in figure 5.6 a and b.

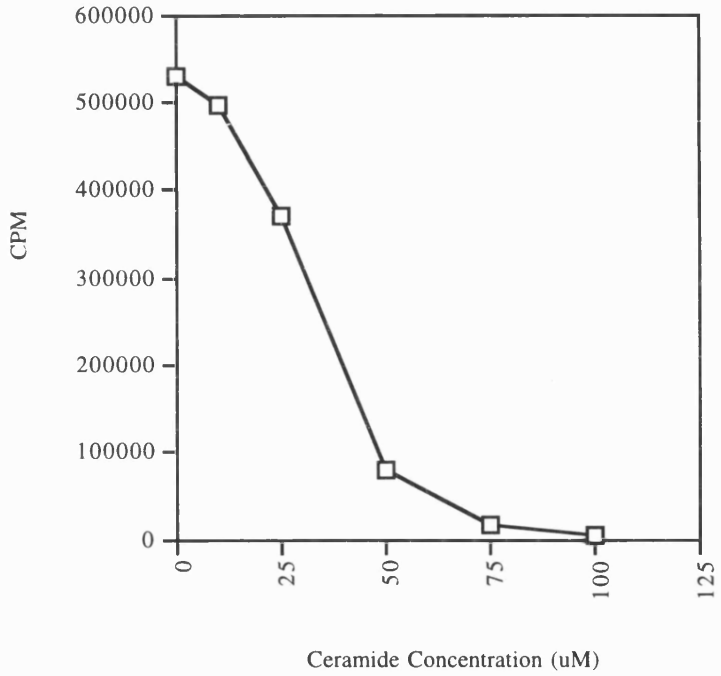


Fig. 5.7a: The Effect of C2 Ceramide on the Proliferation of Jurkats

Jurkat T cells were incubated with or without increasing concentrations of C2 ceramide for 24 hours. All concentrations were equalized for vehicle dilutions. Proliferation was measured by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean of a single representative experiment of n=3 (SDs were less than 5%).

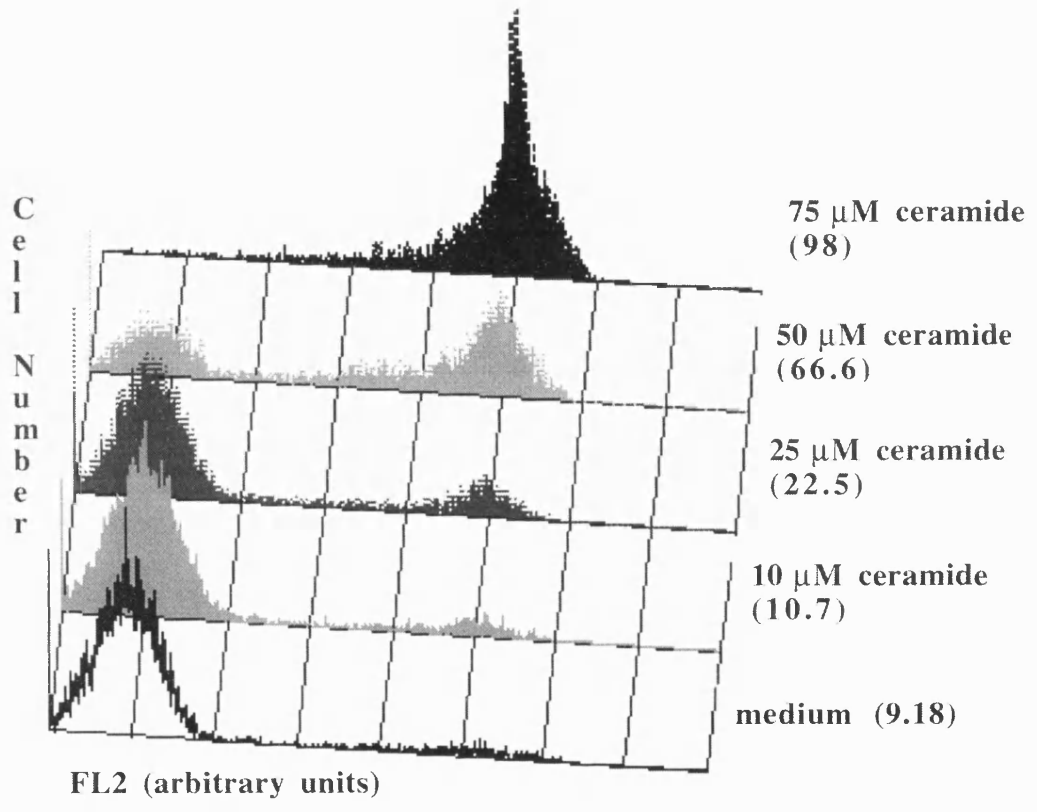


Fig. 5.7b: Effect of C2 Ceramide on the Viability of Jurkats

The effect of C2 ceramide on Jurkat viability after a 24 hour incubation was assessed by FACS analysis. All treatments were equalized for vehicle content at 1% v/v ethanol. Propidium iodide (10μg/ ml) was used to assess cell viability by their ability to exclude the dye. PI uptake was recorded as FL2. Bracketed figures indicate the percentage mortality. These data are paired with figure 5.7a.

5.8-10 Effect of Sphingomyelinase/ C2 Ceramide on the Biology of Costimulated T cells Receiving Different Primary Stimuli

In order to examine the mechanism(s) by which SMase/ C2 ceramide may inhibit the proliferation of αCD3, CD80 costimulated T cells, costimulated T cell proliferation, viability and phenotype were assessed in the presence or absence of SMase/ C2 ceramide. Analysis of the surface expression of proteins associated with T cell activation, CD25 and CD69 (Damle et al., 1992), were used to assess changes in phenotype arising from SMase/ C2 ceramide treatment of T cells. To assess whether or not the primary stimulus required for resting T cell activation and proliferation determined a differential sensitivity to SMase/ C2 ceramide, a parallel series of assays was performed where αCD3 mAb was replaced with PMA as the primary stimulus. CD80 was used as the costimulus in both sets of experiments.

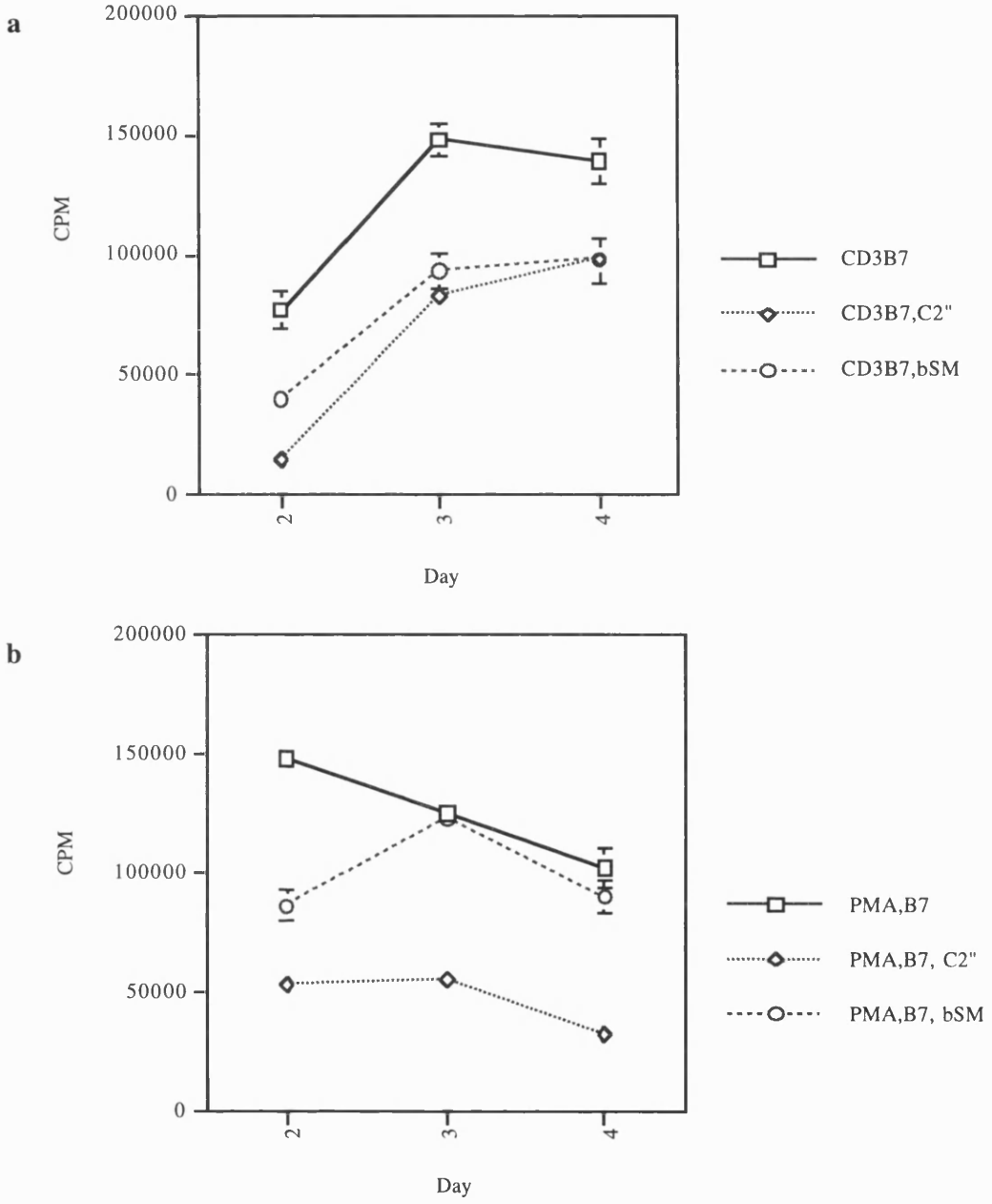
5.8 Proliferation

The effect of using different primary stimuli in CD80 costimulated T cell proliferative responses to SMase/ C2 ceramide was examined. CD80 and αCD3/ PMA costimulated resting T cells were incubated with or without SMase/ C2 ceramide and their proliferation was measured by ^{3}H thymidine incorporation over a time course. Figure 5.8a showed that αCD3, CD80-stimulated T cell proliferation increased up to day 3 and thereafter the level of thymidine incorporation declined slowly. $7.5x10^{-5}$ U/ ml SMase or 30μM C2 ceramide decreased cell proliferation up to day 2. Afterwards the costimulated T cell cultures showed a parallel increase in cell numbers under SMase/ C2 ceramide compared to medium although the maximum levels of proliferation were suppressed due to the initial inhibition.

A maximum level of proliferation under PMA, CD80 costimulation of resting T cells was recorded at day 2. Subsequently proliferation decreased proportionally with time. The maximum level of proliferation induced by αCD3 or PMA was comparable. Control populations, i.e. resting T cells stimulated in the presence of αCD3 mAb, PMA or CD80 alone or αCD3, CHO showed little or no proliferation (<1000 cpm, data not shown). C2 ceramide, over all the days tested, induced a decrease in proliferation of PMA+CD80

stimulated T cells (fig. 5.8b). The effect of SMase on PMA+CD80 stimulated T cells was not consistent with that observed under αCD3, CD80 stimulation and only apparent up to the second day of the proliferation assay. Thereafter the culture, under PMA+CD80 stimulation, was not sensitive to the anti-proliferative effects of SMase.

C2 ceramide consistently inhibited proliferation of resting T cells irrespective of whether the primary stimulus was αCD3 mAb or PMA. PMA stimulated T cells did not show continued sensitivity to inhibition of their proliferation in the presence of SMase, whereas αCD3 stimulated T cells did. Therefore PMA induced a resistance to the effects of SMase but not to the effects of C2 ceramide, whereas αCD3 stimulated T cells which reached peak proliferative levels more slowly than PMA stimulated T cells were susceptible to inhibition of their proliferation in the presence of SMase or C2 ceramide. This observation bears some similarity to the differential sensitivity of blastic or Jurkat T cell proliferation to SMase and C2 ceramide, whereby blasts and Jurkats were insensitive to inhibition of proliferation by SMase (fig. 5.6a and 5.6b respectively) yet sensitive to inhibition by C2 ceramide (fig 5.7a). In summary C2 ceramide inhibited the proliferation of costimulated T cells, irrespective of their primary stimulus up to the second day post stimulation of resting T cells. Thereafter changes in proliferation were parallel to those occurring in the presence of medium. Where PMA, rather than αCD3, was the primary stimulus for proliferation, only a transient decrease in proliferation due to SMase was observed. This was evident on the second day of the assay and thereafter comparable levels of proliferation were shown by SMase and medium in PMA, CD80 stimulated T cells. The inhibition of the proliferative response of costimulated T cells to C2 ceramide was independent of the type of primary stimulus, but the inhibition of proliferation of costimulated T cells by SMase was only maintained where the primary stimulus was αCD3 rather than PMA. Possible reasons for the differential sensitivity of PMA or αCD3 to SMase are presented in the discussion.



5.8: Effect of Sphingomyelinase or C2 Ceramide on the Proliferation of αCD3 or PMA Costimulated Resting T cells

(a) αCD3, CD80 or (b) PMA (40ng/ ml), CD80 resting T cells were incubated in medium or with C2 ceramide (C2, 30μM) or SMase (bSM, $7.5x10^{-5}$ U/ ml) for 4 days. Proliferation was measured from the second to the fourth day by ^{3}H-thymidine incorporation (CPM). Data are the triplicate mean from a representative experiment of n=3 (SD indicated by horizontal bars).

5.9 Viability

To examine whether the reduction of costimulated T cell proliferation by SMase/ C2 ceramide was due to cell death, viability of αCD3 (fig. 5.9a) or PMA (fig. 5.9b) stimulated T cell populations was analyzed in the presence or absence of SMase/ C2 ceramide by a PI exclusion FACS assay. There was no correlation between the effects of SMase/ C2 ceramide on viability with their negative effects on proliferation. In only one case (fig. 5.9biii, day 3) did SMase reduce viability, from 12% without SMase to 27.1% with SMase, and this was not paralleled by an inhibition of proliferation on that day in PMA, CD80 stimulated T cells (fig. 5.8b). Therefore the reductions in proliferation caused by SMase/ C2 ceramide were not an artefact of cell death. It may then be possible to suggest that SMase and C2 ceramide modulate cell division. It may also be possible that they inhibit cellular activation. To examine the latter possibility, the effects of C2 ceramide and SMase were examined on surface markers which were associated with T cell activation.

5.10 CD25 and CD69 Expression

As SMase/ C2 ceramide could inhibit proliferation it may be possible that they down regulate T cell activation. In order to examine this possibility the expression of CD25 and CD69 were examined by FACS analysis over the course of a proliferation assay. Unstimulated T cells or cells stimulated by CD80 with αCD3 or PMA with or without SMase/ C2 ceramide were examined for surface expression of CD25 and CD69 by FACS. Preliminary data (fig. 5.10a+b) illustrates that CD25 and CD69 respectively underwent delayed upregulation due to SMase/ C2 ceramide. CD25 expression was limited at day two by a decrease in the number of cells expressing CD25 which was 33.6% in αCD3, CD80 costimulated T cells and 19 or 20.6% where SMase/ C2 ceramide were added. By day 4 post-stimulation the difference between treatments is less. While all show comparable intensity of expression, there are more cells expressing CD25 in the SMase/ C2 ceramide treated samples.

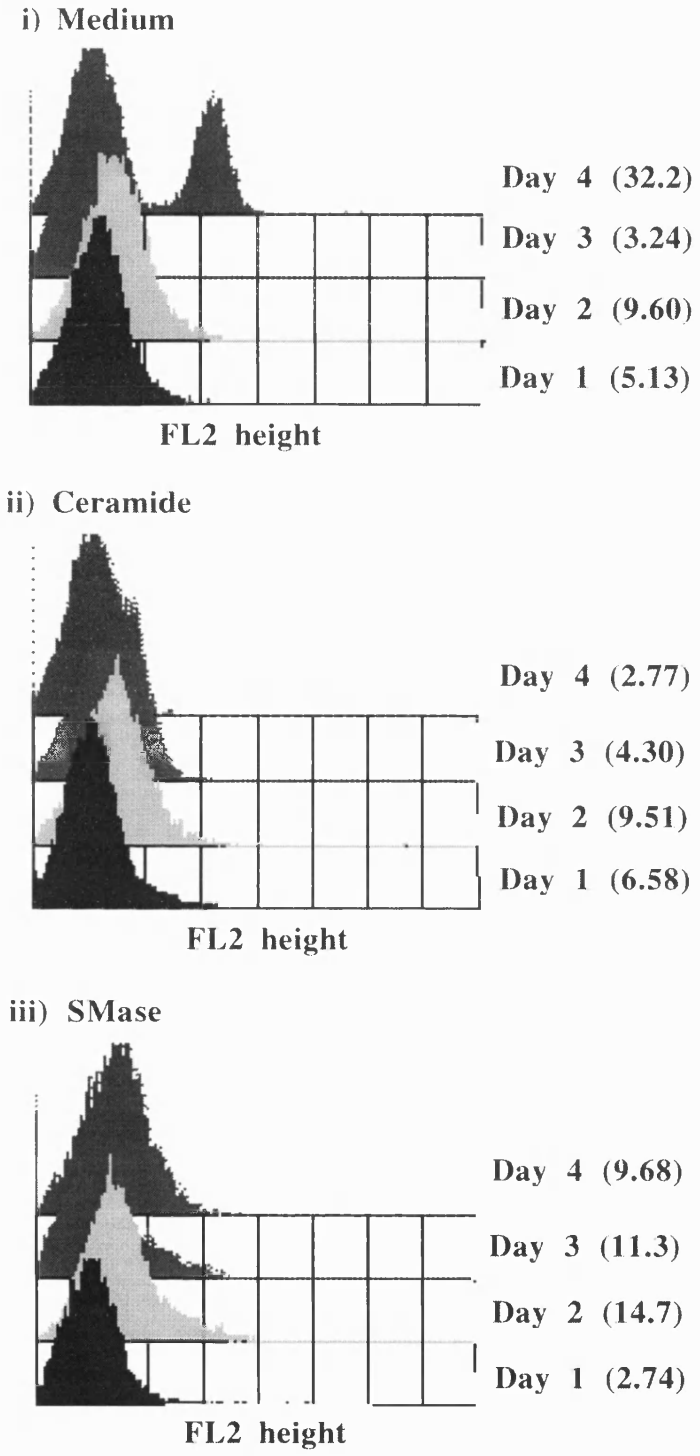


Fig. 5.9a: Effect of Sphingomyelinase or C2 Ceramide on the Viability of Resting T cells Stimulated by αCD3, CD80 Over a Time Course

The viability of αCD3, CD80-stimulated T cells in the presence of i) medium, ii) 30μM ceramide or iii) 7.5 x 10^{-5} U/ ml SMase was assessed by FACs analysis over a 4 day period. Overlapping histograms show levels of propidium iodide (PI, 10μg/ ml, FL2) uptake which is proportional to cell mortality. Figures in brackets indicate the percent of cells showing positive FL2 above control. Data are paired with figure 5.8.

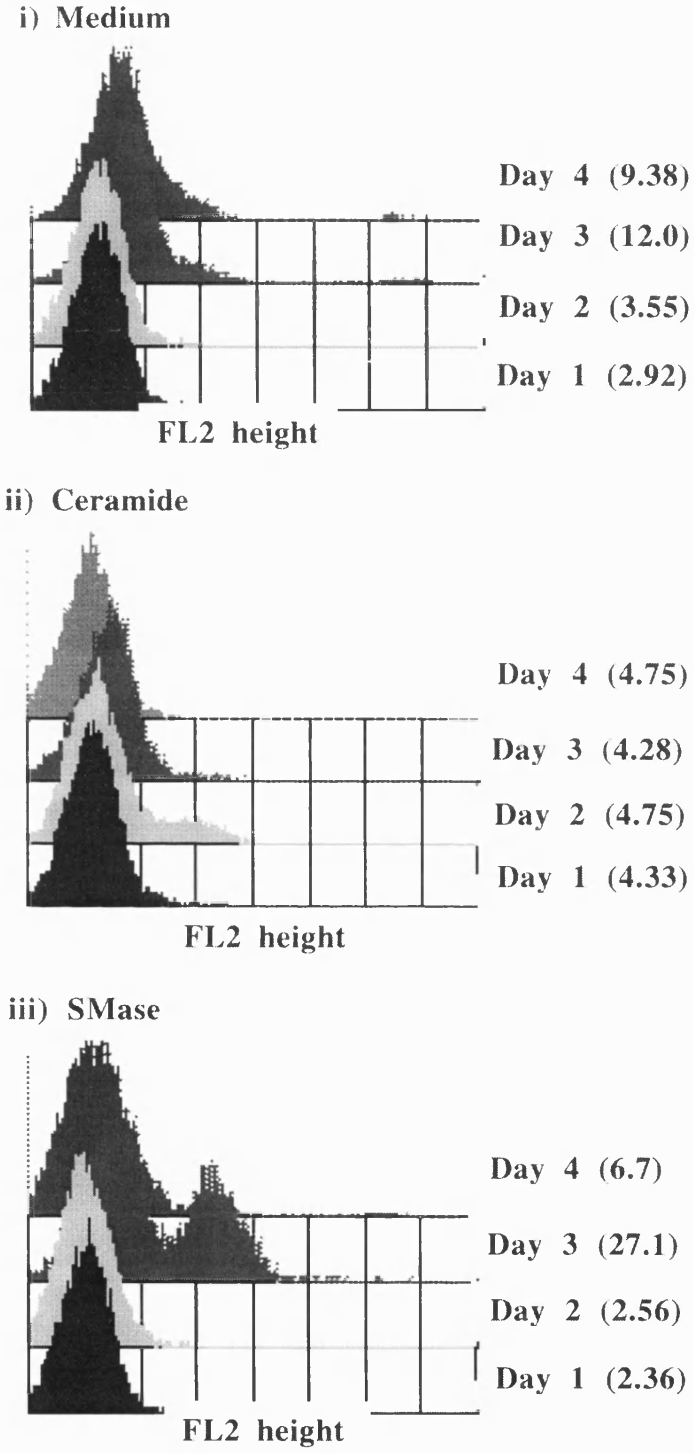


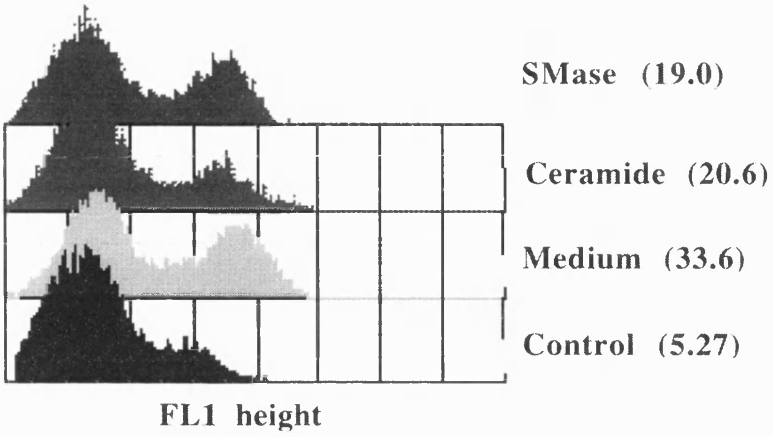
Fig. 5.9b: Effect of Sphingomyelinase or C2 Ceramide on the Viability of Resting T cells Stimulated by PMA, CD80 Over a Time Course

The viability of PMA+ CD80-stimulated T cells in the presence of i) medium, ii) 30μM ceramide or iii) 7.5 x 10^{-5} U/ ml SMase was assessed by FACs analysis over a 4 day period. Overlapping histograms show levels of propidium iodide (PI, 10μg/ ml, FL2) uptake which is proportional to cell mortality. Figures in brackets indicate the percent of cells showing positive FL2 above control. Data are paired with figure 5.8.

CD69 upregulation underwent a similar delay due to SMase/ C2 ceramide whereby at day 1, 55.2% of αCD3, CD80 costimulated T cells expressed CD69, an increase of almost ten fold compared to unstimulated T cells. While the intensity of expression was not affected SMase/ C2 ceramide showed 41.9 and 37.4% of the population to have upregulated CD69 expression. Four days later, more (42.5%) of the C2 ceramide treated cells expressed CD69 and slightly less (26%) of the SMase-treated T cells expressed CD69 than the costimulated T cells which had undergone a decrease in intensity and the number of cells (29.9) showing positive fluorescence relative to the levels observed on day 1.

Figure 5.10c shows the effect of SMase/ C2 ceramide on CD25 and CD69 surface expression under PMA+CD80 stimulation of resting T cells. When PMA was the primary proliferative stimulus both the intensity of expression and the number of cells expressing either CD25 or CD69 was more rapidly upregulated than where αCD3 mAb was the primary stimulus. Nearly all the cells under PMA stimulation (94.5%) showed positive staining for CD25 while only 33.6% of αCD3 mAb stimulated T cells had increased expression. For CD69 expression on day 1, PMA induces over 90% of the cells to express CD69 while when αCD3 mAb was the primary proliferation stimulus, 55.2% of the cells had upregulated expression. Finally while PMA induced a more rapid and profound increase in CD25 and CD69 expression, neither SMase nor C2 ceramide modulated PMA induced changes in expression. PMA appeared to be a more effective stimulus for T cell activation than αCD3 mAb and caused T cells to become insensitive to the effects of SMase on proliferation and SMase/ C2 ceramide on the expression of activation markers CD69 and CD25.

i) CD25 Day 2



ii) CD25 Day 4

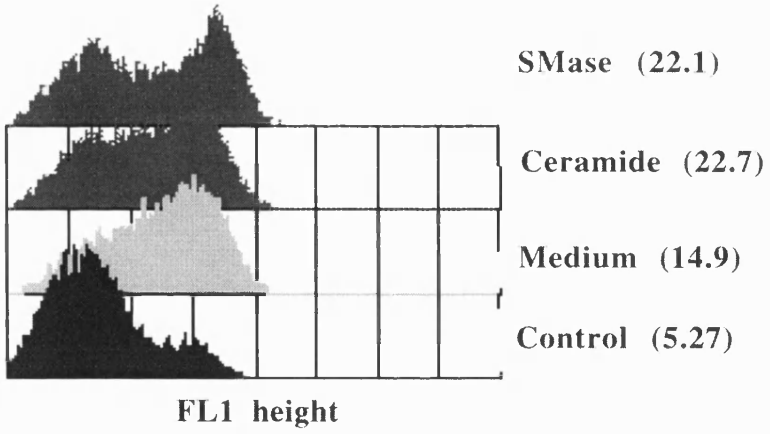
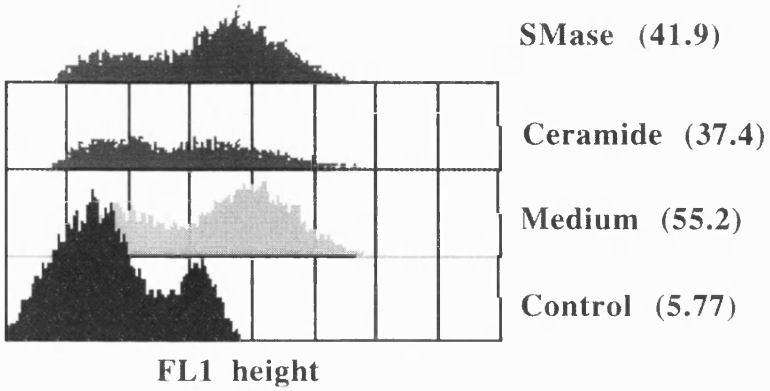


Fig. 5.10a: Effect of SMase/ C2 Ceramide on CD25 Expression on αCD3, CD80 Costimulated T cells

Resting T cells were stimulated with αCD3, CD80 as a stimulus and CD25 surface expression was measured by FACS over a 4 day period post stimulation in the presence/ absence of $7.5x10^{-5}$U/ ml SMase/ 30μM ceramide. Changes in expression are represented by the histograms depicted and the percentage of cells showing positive expression is indicated in brackets. Control is the level of CD25 observed on unstimulated resting T cells. Data are paired with figure 5.8.

i) CD69 Day 1



ii) CD69 Day 4

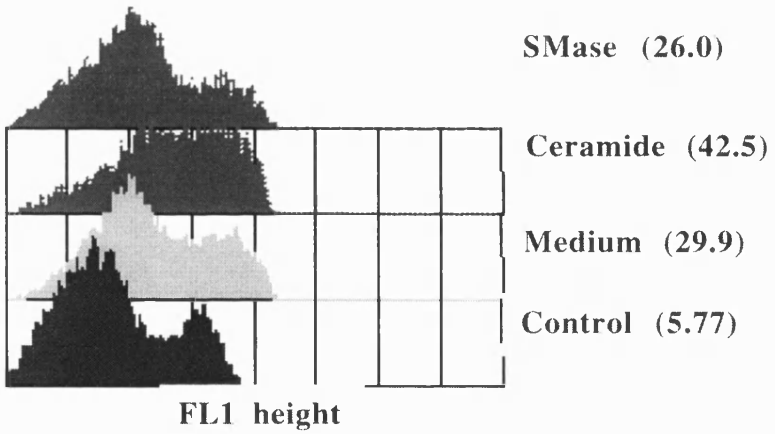


Fig. 5.10b: Effect of SMase/ C2 Ceramide on CD69 Expression on αCD3, CD80 Costimulated T cells

Resting T cells were stimulated with αCD3, CD80 as a stimulus and CD69 surface expression was measured by FACS over a 4 day period post stimulation in the presence/ absence of $7.5x10^{-5}$U/ ml SMase/ 30μM ceramide. Changes in expression are represented by the histograms depicted and the percentage of cells showing positive expression is indicated in brackets. Control is the level of CD69 observed on unstimulated resting T cells. Data are paired with figure 5.8.

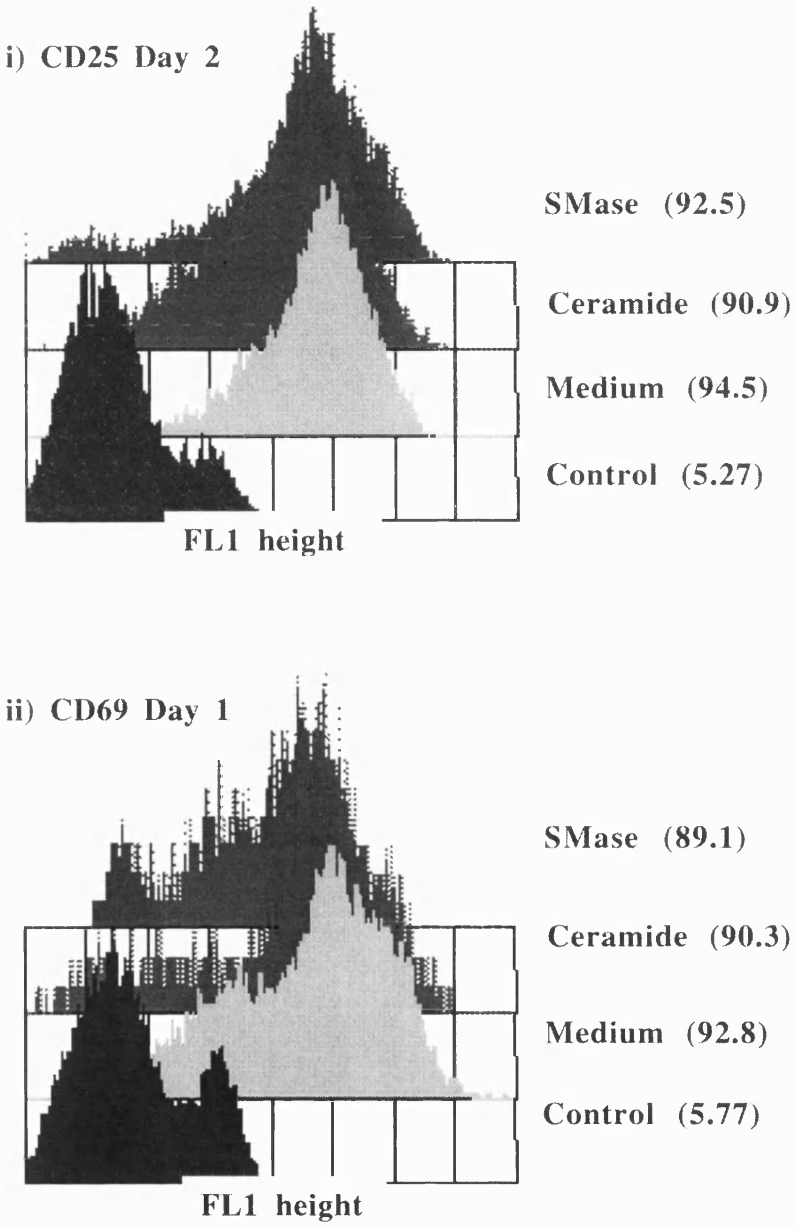


Fig. 5.10c: The Effect of SMase/ C2 Ceramide on CD25 and CD69 Expression in PMA+CD80 Stimulated Resting T cells

Resting T cells were stimulated with PMA, CD80 as a stimulus and CD25 and CD69 surface expression was measured by FACS over a 4 day period post stimulation in the presence/ absence of 7.5×10^{-5}U/ ml SMase/ 30μM C2 ceramide. Changes in expression are represented by the histograms depicted and the percentage of cells showing positive expression is indicated in brackets. Control is the level of CD25 or CD69 observed on unstimulated resting T cells. Data are from a single determination and are paired with figure 5.8.

5.11 Discussion

The experiments conducted in this chapter attempted to analyze the costimulatory requirements of resting human T cells. It was found that resting human T cells could proliferate in response to a primary stimulus of αCD3 mAb or PMA and a costimulus of CD80 (fig. 5.2), but not to either primary stimulus or the costimulus alone. This is in agreement with the experiments of a number of other authors (Ward et al., 1995; Ueda et al., 1995; Hara et al., 1985; Krummel and Allison, 1996; Walunas et al., 1996; Damle et al., 1992; Sansom et al., 1993; Krummel and Allison, 1995) who found CD28 was necessary to costimulate T cell proliferation

The role of SMase and C2 ceramide in costimulation was also examined. Neither SMase nor C2 ceramide could costimulate the proliferation of resting human T cells cultured with αCD3 mAb (fig. 5.2), in contrast to their effect on splenic murine T cells (Boucher et al., 1995; Chan and Ochi, 1995) where a two to five fold increase in proliferation was observed beyond the level achieved by stimulating with αCD3 mAb or PMA alone. This difference in response between murine and human T cells supports the data revealing different biological responses from T cells originating from different species e.g. murine T cells may become activated independent of PI3K (Truitt et al., 1995; Ni et al., 1996) while human T cells have a requirement for PI3K to proliferate and become activated (Ward et al., 1995; Ueda et al., 1995). In our study we observed that in addition to C2 ceramide not costimulating T cell proliferation, it reduced the viability of T cells not receiving costimulation e.g. unstimulated resting T cells (fig. 5.3) and Jurkats (fig. 5.7b) which both showed a dose responsive decrease in viability due to C2 ceramide. Interestingly neither Jurkat or T cell blast viability nor proliferation were affected by SMase (fig. 5.6). Thus T cell responses to SMase and C2 ceramide were not equal where T cells were of an "activated" phenotype. Furthermore a differential sensitivity to a SMase- rather than a C2 ceramide-induced inhibition of proliferation displayed by costimulated T cells was dependent on the primary stimulus. While αCD3, CD80 stimulated T cell proliferation was inhibited by SMase/ C2 ceramide equally (fig. 5.8a) PMA, CD80 costimulated T cell proliferation was only transiently sensitive to inhibition

by SMase whereas C2 ceramide-inhibited proliferation did not recover to the levels obtained with controls (fig. 5.8b).

Human T cells of, or approaching, an activated phenotype appear resistant to a SMase-induced inhibition of proliferation, yet sensitive to C2 ceramide. While T cell blast and Jurkat proliferation and viability were unaffected by SMase, Jurkat viability was markedly decreased by C2 ceramide. In addition the PMA, CD80 but not the αCD3, CD80 costimulated T cells were only transiently sensitive to a SMase-induced inhibition of proliferation. Accordingly when activation marker expression was analyzed, it was found that CD69 and CD25 expression were more rapidly increased where PMA rather than αCD3 mAb was the primary stimulus in a costimulation assay (fig. 5.10). Thus the phenotype of PMA, CD80 stimulated T cells resembled that of an activated cell and as such became less sensitive to SMase as was observed for T cell blasts and Jurkats. This would have the effect of limiting the exposure of PMA, CD80 stimulated T cells to second messengers such as C2 ceramide generated by extracellular enzymes such as SMase (Spence, 1993). Clearly activated T cells are sensitive to C2 ceramide-induced cell death as C2 ceramide was capable of killing Jurkats (fig. 5.7b) and therefore limiting the deleterious effect of agents such as SMase would be beneficial to cell viability. However costimulated T cell viability was not compromised by C2 ceramide, although proliferation was (fig. 5.9). Anti-apoptotic effects due to PMA against ceramide-induced (apoptotic) death or CD28-induced expression of the survival factor bcl-x_L (Boise et al., 1995) may provide the mechanism by which costimulated T cells survive death caused by SMase/ C2 ceramide.

The transient susceptibility of PMA, CD80 stimulated T cells and the resistance of T cells of an activated phenotype e.g. Jurkats and blasts to reductions in proliferation due to SMase may be a mechanism of enhancing cell survival when long term exposure to PMA results in the down regulation of PKC activity (Zhang et al., 1990). When this occurs, an anti-apoptotic effect of PKC would be lost over time (Haimovitz-Friedman et al., 1994) and so the change in susceptibility to SMase may be necessary to maintain cell viability. While PMA may antagonize ceramide mediated apoptosis, it was also apparent

that the viability of PMA, CD80-stimulated T cell cultures was not reduced by C2 ceramide. This would indicate that perhaps CD28 more than TCR derived signals regulate costimulated T cell survival.

Following the observation that a SMase/ C2 ceramide reduction in costimulated T cell proliferation was not simply due to cell death, an assessment of T cell activation state revealed that C2 ceramide and SMase delayed the upregulation of the early activation marker CD69 and the IL2R, CD25. This delay in the activation of T cells was dependent on αCD3 as a primary stimulus, rather than PMA, suggesting substrates for C2 ceramide exist upstream of PKC. One such target of C2 ceramide might be Ras, as dominant negative Ras ablated a ceramide-induced apoptotic response (Gulbins et al., 1995) showing that Ras and ceramide can functionally interact and blocking Ras activity leads to T cell inactivation (Williams, 1996) i.e. lack of IL2 secretion. As Ras interacts with molecules involved in cytoskeletal rearrangement such as Rac1 and Cdc42 (Qiu et al., 1995), modulation of Ras or for example Rac1 or Cdc42 may provide a mechanism for C2 ceramide to inhibit CD69 and CD25 transport to the cell surface.

However as C2 ceramide inhibited costimulated T cell proliferation irrespective of the type of primary stimulus, an additional target for C2 ceramide down stream of PKC seems likely. One element involved in negative regulation of cell cycling is the retinoblastoma protein (RbP) which when dephosphorylated decreases c-Myc levels (Cooper and Whyte, 1989). Concordantly, C2 ceramide has also been demonstrated to limit c-Myc levels (Wolff et al., 1994), an effect prevented by okadaic acid (Wolff et al., 1994). The ceramide activated protein phosphatase, CAPP, is also inhibited by okadaic acid (Dobrowsky and Hannun, 1992) and it may be that C2 ceramide activates CAPP causing the dephosphorylation of RbP thereby limiting cell cycling through decreasing c-Myc levels. Indeed C6 ceramide has been demonstrated to dephosphorylate RbP and induce cell cycle arrest (Dbaibo et al., 1995) and this may be the mechanism by which C2 ceramide inhibits T cell proliferation.

Despite the differences in response of resting T cells to SMase/ C2 ceramide in the presence of CD3 or PKC signals with respect to surface marker expression, there was little difference in the effect of C2 ceramide in its ability to reduce proliferation from either primary stimulus. Therefore while some activation events e.g. upregulation of CD25 may be differentially sensitive to SMase/ C2 ceramide depending upon the primary stimulus delivered, the proliferative response of the T cells did not discriminate between C2 ceramide signals in the presence of CD3 or PKC derived pathways. This might suggest that the proximal signals may be more relevant to activation of T cells and signals distal to PKC control proliferation. Alternatively it may also imply that a target for C2 ceramide regulating T cell proliferation is present in CD28 signal transduction pathways or in both distal TCR pathways and CD28 pathways. To further analyze what role C2 ceramide and SMase may play in T cell signalling, the activation of JNK in resting T cells was examined under various combinations of stimuli as demonstrated in the next chapter.

Chapter 6

Role of Sphingomyelinase and C2 Ceramide in JNK Activation in T cells

The c-Jun terminal kinase (JNK) family members can phosphorylate c-Jun, a component of a heterodimeric transcription factor, AP1, which is involved in activation of the IL2 gene promoter (Granelli-Piperno and Nolan, 1991). JNKs function to stimulate the transcriptional activation of c-Jun by phosphorylation of serine residues (Ser^{63} and Ser^{69}) in the transactivation domain of c-Jun (Faris et al., 1996). c-Jun upregulates its own expression by interacting with the *c-jun* promoter (Faris et al., 1996). In addition JNKs also upregulate c-Fos expression by phosphorylation of the ternary complex factor $p62^{TCF}$ (Elk-1) which binds the *c-fos* promoter (Faris et al., 1996). T cells receiving proliferative and activation stimuli are capable of inducing AP1 formation (Su et al., 1994; Faris et al., 1996; Granelli-Piperno and Nolan, 1991). The available evidence would indicate that JNK is involved in promoting, rather than inhibiting, T cell activation (Faris et al., 1996; Su et al., 1994; Granelli-Piperno and Nolan, 1991) and that the generation of AP1 is jointly dependent on signals derived from TCR and CD28 pathways (Su et al., 1994; Faris et al., 1996; Rincon and Flavell, 1994) to facilitate the translocation of c-Jun from cytoplasm to nucleus (Su et al., 1994; Minden et al., 1995). In contrast JNK may also be activated by ligation of TNFR1, a receptor delivering apoptotic signals to cells (Verheij et al., 1996). Therefore the role of JNK in cells is unclear.

Ceramide has also been shown to induce JNK activation (Verheij et al., 1996; Gulbins et al., 1995; Kyriakis et al., 1994). Due to the role of AP1 in promoting IL2 transcription (Granelli-Piperno and Nolan, 1991) and the activation of JNK by cellular stress (Verheij et al., 1996), JNK may be a sensitive and pivotal element in determining cellular behaviour. While SMase has been suggested to be an effector of CD28 costimulatory function in blasts and Jurkats (Boucher et al., 1995; Chan and Ochi, 1995) it was not costimulatory in resting T cells (Chapter 5, fig. 5.2a). Therefore a study was designed to examine whether a SMase or C2 ceramide derived modulation of JNK activity might provide an explanation of the mechanism by which SMase/ C2 ceramide could alter T cell responses.

6.1 Induction of GST.c-Jun Expression

In order to measure JNK activity, a substrate for JNK was expressed in a bacteria (E. coli) using an IPTG-sensitive promoter to express a GST.c-Jun fusion construct. The c-Jun residues provided the substrate for JNK and the GST domain provided a mechanism to immobilise c-Jun by a GST interaction with glutathione agarose beads (GAB). Following growth of the cells with or without IPTG, the cells were harvested, lysed and proteins separated by SDS-PAGE were visualized by Coomassie staining. Figure 6.1 demonstrated that the GST.c-Jun vector could be induced to upregulate expression of a protein of ~36 kDa when the vector promoter was stimulated by IPTG corresponding to the predicted molecular weight of GST.c-Jun. Subsequently this protein was immobilized on GAB and so an immobilised substrate for the assessment of JNK activity was prepared.

6.2 Ability of C2 Ceramide to Activate JNK in Jurkats

In order to determine whether it was possible to detect JNK activation using the GST.c-Jun fusion protein, Jurkats were incubated for 20 mins with or without C2 ceramide or PMA/ ionomycin stimuli. Figure 6.2a illustrates the effect of PMA/ ionomycin or C2 ceramide-stimulation of Jurkats on the phosphorylation of c-Jun. PMA/ ionomycin stimulated JNK activity, and this supports previous findings (Su et al., 1994), where PMA and the calcium ionophore, A23187 were used in the report mentioned. Figure 6.2 also demonstrated that C2 ceramide, in a dose dependent manner, showed increasing stimulation of JNK activity independent of its vehicle. Protein loading controls (fig. 6.2b) indicated equal loading between wells, except for the 'OLD' beads which had a lower titre of protein than the NEW beads. This would account for the lower level of c-Jun phosphorylation recorded in lanes 1+2 compared to 3+4 in figure 6.3a. It was possible to conclude that a stimulation-sensitive JNK assay had been established.

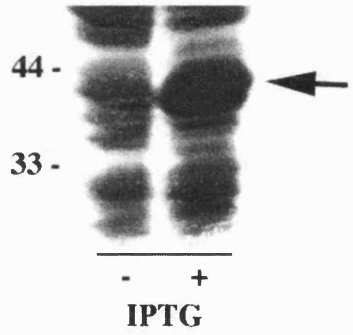
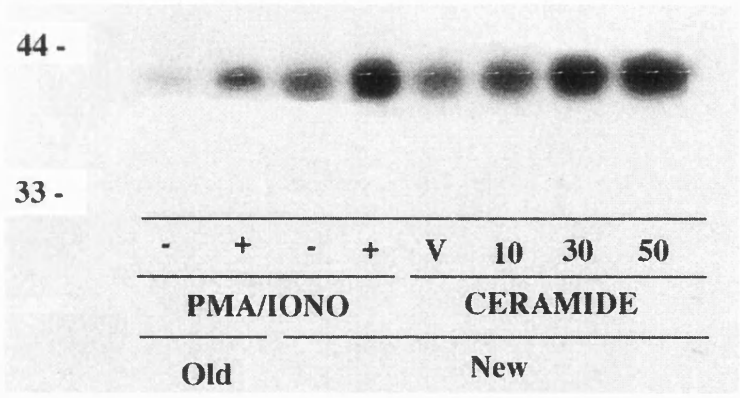


Fig. 6.1: Ability of IPTG to Induce GST.c-Jun Expression

E.coli cells containing an IPTG-inducible GST.c-Jun PGEX expression construct were incubated with/ without 400μM IPTG for 4 hours. Cell lysates were separated by SDS-PAGE and proteins were visualized by Coomassie staining. Arrow indicates a ~36 kDa protein found to be upregulated under IPTG induction and corresponding to the predicted molecular weight of GST.c-Jun.

a



b

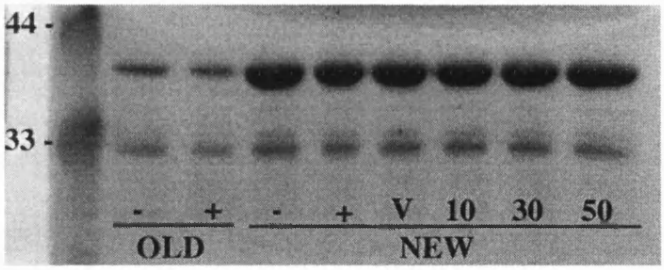


Fig. 6.2: Activation of JNK by PMA/ Ionomycin or Ceramide

a) Jurkats ($5x10^6$ cells/ lane) were analyzed for JNK activity under stimulation through medium (-); PMA(40ng/ ml) and ionomycin (1µM) (+); vehicle (ethanol, V) or various concentrations of ceramide (10-50µM). All lanes in the ceramide stimulations were equalized for ethanol content to be equivalent to 50µM ceramide. b) Lanes as (a), Coomassie blue stained gel, control for protein loading.

6.3 Ability of Sphingomyelinase or C2 Ceramide to Modulate JNK Activity in Stimulated Human Resting T cells

In an attempt to account for the effects of SMase and C2 ceramide in limiting the proliferation of primary T cells, JNK activity in human resting T cells was assessed. Resting T cells were left unstimulated or stimulated with CD80 and/ or PMA or αCD3 mAb. The effects of SMase, C2 ceramide or its vehicle, ethanol compared to medium on JNK activation were assessed for each of the treatments in a 20 mins stimulation at 37°C of resting T cells. Figure 6.3a+b illustrates the degree of c-Jun phosphorylation recorded (upper panels) and the levels of protein associated with each sample (lower panels). It may be seen that the greatest stimulation of JNK activity was attributable to PMA (fig. 6.3b, upper panel, lane 1 to 8). Figure 6.3a (upper panel) shows that neither αCD3 mAb (lane 9) nor CD80 alone (lane 5) stimulated JNK activation beyond the level obtained in unstimulated T cells in medium (lane 1). Small variations in the level of c-Jun phosphorylation were apparent although these were paralleled by variation in protein loading (lower panel fig. 6.3a). Surprisingly in contrast to the effect of C2 ceramide in Jurkats, SMase/ C2 ceramide had little effect on modulating the level of JNK activation in each treatment relative to medium in resting T cells. A combination of CD80 and αCD3 mAb stimulation lead to a greater activation of JNK, supporting the idea that either signal alone was an insufficient activation and / or proliferation stimulus (Thompson et al., 1989; Damle et al., 1992), although the greater degree of JNK activation may be an artefact of protein loading. By far the strongest stimulus for JNK activation was PMA (fig. 6.3b) which seemed insensitive to modulation by other stimuli including CD80. At 40ng/ ml the concentration of PMA may have been high enough to maximally activate JNK as in a previous report PMA at 10ng/ ml required additional stimuli such as αCD28 mAb to activate JNK in Jurkats (Su et al., 1994). In conclusion there was no correlation between the effect of SMase/ C2 ceramide on modulation of JNK activity and their effect on inhibition of proliferation in costimulated T cells. Costimulation of T cells resulted in a possibly greater activation of JNK by αCD3, CD80 stimulation while PMA strongly stimulated JNK activation independent of additional stimuli.

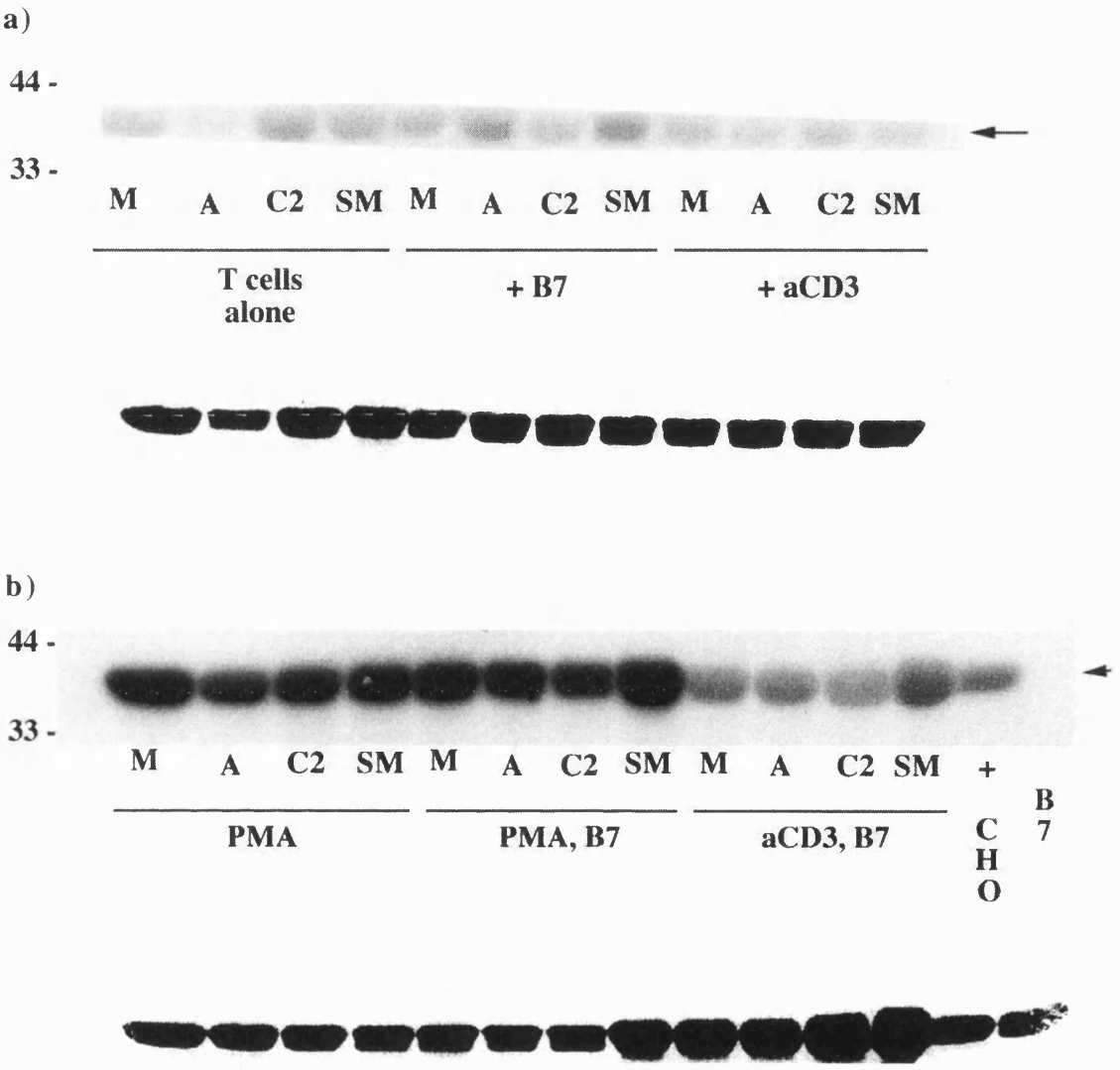


Fig. 6.3: Effect of Sphingomyelinase or C2 Ceramide on c-Jun Phosphorylation in Resting T cells Stimulated with CD80 and αCD3/ PMA

Resting T cells ($1x10^7$ cells/ lane) were incubated for 20 min at 37°C with a) medium (alone), fixed $CD80^+$CHOs ($3.3x10^6$ / lane, B7) or αCD3 mAb (1/100 dilution from ascites) or b) PMA (40ng/ ml), PMA-B7 , αCD3-B7 or non-transfected fixed CHOs (CHO). Each treatment (except CHO and B7 alone) was carried out in quadruplicate with one point of each being incubated with medium (M), 30μM ceramide (C2), 0.3% v/v ethanol (A) or 7.5×10^{-5} U/ ml SMase (SM). b, last lane, fixed $B7^+$CHOs alone were assessed for JNK activity. JNK activity, represented by the degree of c-Jun phosphorylation (see arrow heads), was measured following incubation of cell lysates with immobilized GST.c-Jun and IVK assay. Lower panels in a and b indicate controls for protein loading as measured by Coomassie Blue staining.

6.4 Discussion

An examination of the effects of SMase/ C2 ceramide on JNK activation in costimulated T cells was made. Following the preparation of a JNK substrate and successful establishment of an assay to measure JNK activation, it was found that although JNK could be activated by a combination of αCD3, CD80 signals (fig. 6.3), neither SMase nor C2 ceramide modulated the degree to which JNK was activated. This was surprising because in Jurkats, C2 ceramide, without additional stimuli, proved a strong stimulus for JNK activation (fig. 6.2). By contrast C2 ceramide altered the phosphorylation of c-Jun little in resting T cells under a variety of signals associated with proliferation and in unstimulated T cells, suggesting that either the effects of SMase/ C2 ceramide in T cells were negatively regulated relative to modulation in Jurkats or alternatively resting T cells lacked a signalling element (or critical titre thereof) present in Jurkats. Despite this possibility PMA was a strong stimulus for JNK activation in resting T cells (fig. 6.3b) in agreement with a PMA-induced activation of JNK in primary hepatocytes (Minden et al., 1995) and PMA+ionomycin stimulated JNK activation in Jurkats (fig. 6.2) and (Su et al., 1994). One study demonstrated that suboptimal levels of PMA (10ng/ ml) synergize with αCD28 mAb in stimulation of JNK activity (Su et al., 1994) indicating that the strong stimulation observed in this chapter was likely due to a dose effect. Alternatively the difference in the level of JNK activity observed with PMA compared to αCD3 mAb may be due to negative regulatory elements upstream of PKC and downstream of CD3.

Some data have suggested that CD28 untilizes ASMase/ C2 ceramide as a costimulatory signal (Boucher et al., 1995; Chan and Ochi, 1995) for T cell proliferation and NFkB activation. If CD28 does transduce costimulatory signals through SMase then the stimulation of CD28 and CD3 or SMase/ C2 ceramide and CD3 would be expected to have a similar effect on JNK activation. No modulation of c-Jun phosphorylation due to SMase/ C2 ceramide was observed in resting human T cells (fig. 6.3). In addition as demonstrated in chapter 5, figure 5.2, SMase/ C2 ceramide did not replace CD28 as a costimulus for T cell proliferation. These observations would lead to the suggestion that SMase/ C2 ceramide modulate alternative substrates to inhibit T cell proliferation. In fact

the JNK family member p38, independent of c-Jun phosphorylation, induced *c-jun* expression in response to UV-irradiation (Hazzalin et al., 1996). As SMase and C2 ceramide have been implicated in stress responses e.g. facilitating apoptosis following ligation of TNFR (Higuchi et al., 1996) or in stress-induced apoptosis (Verheij et al., 1996) it may be suggested that C2 ceramide preferentially activates the stress responsive JNK family member p38, while another member of the JNK family p54 JNK, may be a substrate for effectors of proliferative stimuli. For example p54 JNK activity correlated closely with c-Jun associated kinase activity in U937 cells (Kyriakis et al., 1994). It would be interesting to examine whether the response of p38 and p54 JNK isolated from resting T cells following stimulation by C2 ceramide revealed differences in activation.

A mechanism by which C2 ceramide may decrease proliferation through cell death, as in the case of Jurkats (Chapter 5, figure 5.7) is by apoptosis. Although costimulated T cell cultures did not show a reduction in viability due to SMase/ C2 ceramide, it is possible that the assay was measuring the viability of C2 ceramide-insensitive 'survivors'. Accordingly apoptosis through Fas- or TNFR-stimulation was paralleled by an increase in SMase activity and Jurkats were sensitive to ceramide-induced apoptosis (Cifone et al., 1993). This may occur through Ras as dominant negative mutant, N17 Ras, decreased Fas or ceramide induced apoptosis, indicating a common apoptotic pathway is shared by ceramide and Fas (Gulbins et al., 1995). Alternatively a study on TNFR1 identified different effectors for three functions arising from TNFR ligation (Liu et al., 1996). The Fas-associated protein with death-domain, FADD, was found to mediate apoptosis independent of JNK activity (Liu et al., 1996). Similarly we observed that ceramide did not stimulate JNK activation in resting T cells (fig. 6.3), but did decrease the proliferation of costimulated T cells (chapter 5, fig. 5.2). A complex of the TNFR1 and the TNFR1-associated death domain protein, TRADD, associated with two other transducers. TNFR-associated protein 2, TRAF2, and receptor-interacting protein, RIP, were found to mediate NFkB and JNK activation although the mechanism of JNK activation was not known. Various dominant negative mutants elucidated that these three events could occur independently of one another and expression of the NFkB component c-Rel, and to a lesser extent RelA, had an anti-apoptotic effect (Liu et al., 1996). In agreement with this

was the survival of costimulated T cells (chapter 5, fig. 5.4), unlike unstimulated (fig. 5.3) or activated T cells (fig. 5.7), which were insensitive to ceramide-induced apoptosis possibly through an anti-apoptotic effect of CD28-induced NFkB nuclear translocation (Granelli-Piperno and Nolan, 1991). Thus it could be hypothesised that receptors which functionally associate with SMase determine the final outcome of increased intracellular levels of ceramide or more specifically that CD28 possibly diverts C2 ceramide into inducing NFkB translocation, thus protecting the cell from apoptotic effects which C2 ceramide may otherwise induce.

This study established an assay to assess JNK activation. JNK was found to be activated beyond the levels observed in unstimulated T cells by either PMA alone or possibly under costimulation by αCD3, CD80. Surprisingly despite the modulation of activation markers CD25 and CD69 demonstrated in the previous chapter (fig. 5.10), neither SMase nor C2 ceramide limited or modulated JNK activity in resting T cells. Paradoxically C2 ceramide effected a far greater stimulation of JNK activity in Jurkats indicating stage specific differences in the regulation of pathways between ceramide targets and JNK activation. Therefore it may be suggested that a ceramide induced activation of JNK in Jurkats partially accounts for its costimulatory capacity in activated T cells such as demonstrated in murine splenocytes (Boucher et al., 1995; Chan and Ochi, 1995). Accordingly data from other studies have indicated synergistic activation of JNK following ligation of CD3 and CD28 in Jurkats (Su et al., 1994; Faris et al., 1996) and furthermore only under multivalent crosslinking of CD28 was c-Jun expression increased, while there was no evidence of synergy between CD3 and CD28 derived signals in upregulating c-Jun expression in resting murine T cells (Chatta et al., 1994). These data suggest that c-Jun phosphorylation and expression, contributing to AP1 formation, is more highly regulated in resting T cells than Jurkats. What is evident from these studies is that it is not clear which factors determine the context of signals such as those generated by SMase/ C2 ceramide in deciding cell fate. While some clues have been provided e.g. from N17 Ras mutants in Fas signalling, and NFkB activation, it remains to be determined at what stage, if at all, different ceramide-induced signalling pathways relate to signals from receptors associated with anti-apoptotic, proliferative and gene

activation effects. These may be at a proximal stage in signalling e.g. Ras or a distal stage e.g. NFkB, *c-jun* expression or through regulatory feedback mechanisms.

Chapter 7

Conclusion

An assessment of the role of the signal transduction elements $p59^{fyn}$, $p56^{lck}$, $p70^{ZAP70}$ and sphingomyelinase which are putatively associated with CD28-mediated costimulation of resting T cell activation was made in this study. In order to analyze the individual contribution of each of the PTKs to CD28 signalling, cell models were prepared of CHO cells expressing CD28 and either fyn, lck or ZAP70. The responses of PTK transfectants to stimulation by CD80 , a natural ligand of CD28, were assessed. It was found that in CD28 immunoprecipitates of stimulated cells the PTKs caused little modulation of tyrosine phosphorylation in response to CD80. However analysis of overall phosphorylation revealed greater changes. Fyn and lck could increase the phosphorylation of molecules likely to be CD28 and possibly PI3K, whereas ZAP70 could not, in response to a CD80-CD28 interaction. It may be concluded that CD28 showed selectivity in activation of distinct families of PTKs and that the ligation of CD28 by CD80 resulted in the activation of lck and fyn. Furthermore while the tyrosine phosphorylation of CD28 and associated molecules was apparently not influenced to a large extent by fyn or lck, tyrosine/ serine/ threonine phosphorylation was sensitive to, and increased by, the action of fyn and lck. In light of the marked increase in phosphorylation of CD28 immunoprecipitates by fyn and lck, it is probable that other kinases are recruited to CD28 through lck and fyn-mediated interactions if the small changes in tyrosine phosphorylation were not an artefact of the primary antibody used in Western blotting. The identity of such kinases are not known although they are unlikely to be either PI3K or PKC because inhibition of their activity by wortmannin and Ro-31 respectively did not alter the phosphorylation of CD28 (Parry et al., 1997). Therefore it still remains an open question which kinase facilitates the profound alteration in phosphorylation of CD28. Further work on the role of fyn and lck in recruitment of, or interaction with, adapter molecules such as p62 (Nunes et al., 1996) and GRB2 (Schneider et al., 1995a) shown to associate with CD28 following respectively CD80 or αCD28 mAb stimulation may be helpful.

Although fyn and lck delivered similar modulatory signals to CD28, they induced a different level of phosphorylation of CD28 immunoprecipitates. As lck was expressed to a lower level than fyn in transfectants relative to the level found in Jurkats, it would be

possible to conclude that the lck-induced greater levels of phosphorylation of CD28 immunoprecipitates, demonstrated lck to be more capable of transducing CD28-associated signals than fyn in CHO transfectants. This conclusion is supported by one study also showing that greater phosphorylation of CD28 was attributable to lck rather than fyn (Raab et al., 1995) and other data ascribing fyn a negative role in T cell function. For example in anergized T cells fyn associates with TCRζ and subsequent costimulation causes fyn dissociation and replacement by lck (Boussiotis et al., 1996). Support for a divergent function between fyn and lck may be deduced from data demonstrating fyn -ve cells to be resistant to Fas-induced death (Atkinson et al., 1996) while the lck -ve Jurkat line, JCam1, was not (Gonzalez-Garcia et al., 1997). Therefore fyn, not lck, transduces apoptotic signals from Fas while fyn is also capable of association with Fas (Atkinson et al., 1996). In conclusion the roles of fyn and lck appear to be different and this may be reflected in the preferential activation or recruitment of lck above fyn by CD28 which results in lck facilitating greater phosphorylation responses from CD28 following CD80 stimulation which was observed in this study.

The role of SMase in CD28 signalling was also assessed. Initially it was noted that CD80-stimulated CD28 activated endogenous ASMase activity independently from T cell PTKs. Therefore the contribution ASMase might make to CD28 signalling could be different from that transduced by activation of lck or fyn. In agreement with the paradigm that resting T cells require two signals to proliferate and become activated, this study demonstrated that together αCD3 mAb and CD80 stimulation of resting T cells induced their proliferation while neither stimulus alone could induce proliferation. However despite the reported costimulatory ability of ASMase/ ceramide (Boucher et al., 1995; Chan and Ochi, 1995), we found resting T cell proliferation was not costimulated by SMase or either of the products of sphingomyelin hydrolysis i.e. C2 ceramide or phosphocholine. Therefore in resting human T cells, unlike splenic murine T cells, SMase/ C2 ceramide were not sufficient as a costimulatory stimulus.

The effect of SMase, C2 ceramide and phosphocholine on costimulated T cell proliferation was analyzed in order to assess whether they modulate costimulation. It was observed that SMase/ C2 ceramide but not phosphocholine reduced costimulated resting T cell proliferation. This occurred without an apparent increase in cell death indicating that the reduction in proliferation was not simply due to a fatal perturbation of the plasma membrane due to the action of hydrolytic SMase on membrane localized sphingomyelin nor disruption of the membrane from the passage of C2 ceramide across the membrane. Therefore SMase/ C2 ceramide had the capability to inhibit costimulated T cell proliferation or alternatively to mediate the death of a susceptible proportion of the T cell population.

When the role of SMase in modulating 'activated' T cell proliferation was analyzed, surprisingly it was found to have no effect on the proliferation nor viability of T cell blasts and Jurkats. This would indicate that SMase has stage specific effects and only inhibits the response of resting T cells. In support of this conclusion is the transient sensitivity to SMase of resting T cells costimulated by PMA, CD80. Accordingly the rapid upregulation of the early activation marker CD69 and the later activation marker CD25 when PMA was the primary proliferation stimulus indicated that PMA, CD80 costimulation lead to those T cells gaining an activated phenotype. Therefore in a similar manner to Jurkats and T cell blasts, when PMA, CD80 costimulated resting T cells showed maximum expression of CD69, they became insensitive to SMase-derived inhibition of their proliferation.

Conversely the inhibition of Jurkat or costimulated resting T cells proliferation in the presence of C2 ceramide was not transient. In fact C2 ceramide decreased Jurkat or unstimulated resting T cell viability and in Jurkats the cell death caused by C2 ceramide was proportional to the reduced proliferation levels. The lack of sensitivity to SMase of activated T cells and the contrasting sensitivity to C2 ceramide by both activated and resting T cells leads to the hypothesis that activated T cells become resistant to SMase to limit the deleterious affects of C2 ceramide. In the case of Jurkats this is of particular importance as unlike costimulated T cells, Jurkats die in the presence of C2 ceramide. A

mechanism for activated T cells to become SMase-insensitive is not known, although it may be at the level of a change in membrane composition. It could be suggested that the survival of costimulated T cells, even of an activated phenotype i.e. PMA, CD80 stimulated resting T cells, was due to the induction of survival factors such as bcl-X_L by CD28 (Boise et al., 1995; Mueller et al., 1996) whereas Jurkats and unstimulated resting T cells die in response to C2 ceramide, possibly through apoptosis, due to a lack of survival factors.

In a comparison of the effect of either αCD3 mAb or PMA as a primary stimulus on the response of costimulated T cells to SMase/ C2 ceramide, a delay in upregulation of CD69 and CD25 expression was only apparent where αCD3 mAb was the primary stimulus. This demonstrates that SMase/ C2 ceramide modulate not only the proliferative responses of T cells but also those associated with T cell activation. In addition the insensitivity of PMA stimulated T cells leads to the conclusion that elements between CD3 and PMA act as targets for C2 ceramide. A TCR stimulated Ras activation or rise in $Ca^{2+}i$ may represent targets of C2 ceramide. Indeed dominant negative Ras can inhibit ceramide-induced apoptosis (Gulbins et al., 1995) and CTLA4, which like C2 ceramide, inhibits CD25 and CD69 expression, as well as IL2 secretion (Krummel and Allison, 1996), shows increased surface expression due to raised $Ca^{2+}i$ levels (Linsley et al., 1996). If C2 ceramide were involved in either Ras-induced apoptosis or $Ca^{2+}i$ mobilization, it would provide a mechanism for C2 ceramide to limit T cell activation.

Despite the dependency of SMase on the primary stimulus to determine the longevity of its effect on inhibition of costimulated proliferation, there was no difference in the inhibition of proliferation where C2 ceramide was included in culture. PMA/ αCD3 mAb- CD80 stimulated proliferation of resting T cells was inhibited equally by C2 ceramide. Therefore targets of C2 ceramide which facilitate the inhibition of proliferation are distal to PKC in the costimulatory pathway and separate from the signalling element which SMase/ C2 ceramide modulates in delaying activation marker upregulation. A possible target for C2 ceramide which is involved in activation of the IL2 gene by formation of a heterodimeric transcription factor, AP1, is c-Jun (Granelli-Piperno and

Nolan, 1991). When c-Jun phosphorylation was examined, it was found to be unaffected by SMase/ C2 ceramide although PMA or possibly αCD3, CD80 could induce its phosphorylation. Thus c-Jun did not represent a target for the C2 ceramide-induced inhibition of proliferation and neither SMase/ C2 ceramide inhibit the activation of JNK. Thus alternative targets for SMase/ C2 ceramide, distal to PKC activation, exist which inhibit proliferation.

In conclusion despite intensive efforts extended by many laboratories there still remains, due a highly complex system of regulating T cell immune responses, many questions regarding the mechanism by which CD28 transduces its costimulatory signals. Determination of effector function in CD28 signalling is complicated by the existence of elements with apparent functional redundancy e.g. the ligands CD80 and CD86, the use of lck and fyn by CD28 and by effectors with altered function dependent on the activation state of a cell e.g. PI3K is not required for IL2 production in T cell blasts while it is in resting T cells (Ueda et al., 1995). These issues may be resolved by adopting a matrix approach to experiments to determine which of the possible interactions involved in CD28 costimulatory function occur. Rather than mitogenic stimulation, where little is known about the receptor-specificity of the mitogen, rigorous definition of the activation status of the cells and stimulation through carefully defined and specifically relevant receptors may, in the long term, be more informative.

7.1 Future Work

The data presented here provide a number of areas for further investigation. For example lck rather than fyn may be a more significant effector in CD28 proximal signalling as this study demonstrated a greater capability of lck, compared to fyn, to phosphorylate CD28 immunoprecipitates. Thin layer chromatography may be utilized to determine whether fyn and lck differentially phosphorylate CD28 or its substrates, possibly facilitating different outcomes following recruitment and activation of the two kinases to CD28. Furthermore as CD28 cycles between the plasma membrane and lysosomes (Cefai et al.,

1998), the latter may represent a signalling site for CD28. It would be interesting to determine whether fyn or lck are recruited to lysosomes and whether ligation of CD28 induces preferential recruitment of one kinase more than another. This may be determined by Western blotting CD28 immunoprecipitates from cellular lysates and lysosomal fractions. Increasing amounts of data have linked CD28 signalling with association with adapter molecules eg p62 (Nunes et al., 1996) , p100 Vav (Nunes et al., 1994) and small G proteins Cdc42, Rac (Kaga et al., 1998a; Kaga et al., 1998b). These cellular effectors may provide a mechanism by which fyn and lck have putatively divergent function in CD28 signalling and IVK analysis of PTK immunoprecipitates from CD28 immunoprecipitates may be sensitive enough to reveal putative differences in utilization of very early signalling molecules following ligation of CD28. Both lysates and lysosomes might be analyzed for differences in Cdc42, Rac and p62 localization which would indicate the principle signalling location and whether there may be a possible interaction with ASMase.

The paradigm of CD28 signalling through PI3K has recently gained an unexpected twist in that much CD28 which is lysosomally targeted associates with PI3K (Cefai et al., 1998). However PI3K has homology with a yeast protein involved in cytoskeletal rearrangements, TOR2 (Kunz et al., 1993) and therefore this may imply that PI3K functions partially as an effector involved in translocating CD28 to lysosomes. Therefore the role of PI3K in CD28 signalling may be two fold. The first function would be following activation, D3-phosphoinositol lipids from PI3K have been shown to activate distal signalling effectors eg PKB (Burgering and Coffer, 1995; Franke et al., 1995) and secondly PI3K may be involved in targeting CD28 to a putative signalling site i.e. lysosomes (Cefai et al., 1998).

C2 ceramide exerts different effects on T cell biology dependent on the species from which the T cell is derived, the activation state of the T cell or additional stimuli the T cell receives. On resting human T cells C2 ceramide appears to exert three effects. The first which is apparent in T cells not receiving costimulation is death, which may be assessed as apoptotic by analysing genomic DNA for laddering, a characteristic typical of

apoptosis following DNA digestion in apoptotic cells. A second effect is an apparent inhibition of proliferation of costimulated T cells. This may be the result of death in a susceptible proportion of the T cell population whereby if this were the mechanism accounting for reduced proliferation in ceramide treated cells, the susceptible sets might be identified by FACS analysis of TCR Vβ regions for viability following costimulation in the presence or absence of ceramide. Alternatively C6 ceramide has been demonstrated to inhibit proliferation by dephosphorylating RbP (Dbaibo et al., 1995) and therefore immunoprecipitation of Rb from costimulated T cells following ceramide treatment may reveal RbP as a target for ceramide. The third effect of ceramide was to delay the activation of T cells costimulated by αCD3, CD80, but not by PMA, CD80 implying that ceramide sensitive targets exist between the TCR and PKC. Accordingly there have been reports of functional associations between Ras and ceramide resulting in apoptosis (Gulbins et al., 1995); PI3K and ceramide share some substrates eg PKCζ (Pushkareva et al., 1995; Nakanishi et al., 1993) and Ras and PI3K may activate each other (Qiu et al., 1995; Rodriguez-Viciana et al., 1994). Therefore there is a possibility that each of these three signalling effectors impinge upon each others functions in T cells, although how is unclear. Perhaps the small G proteins Cdc42 and Rac are involved in transducing signals between them. An anti-apoptotic function for CD28 might be dependent upon its translocation to lysosomes. Interaction with ASMase, following association with PI3K (Cefai et al., 1998), may divert ceramide into anti-apoptotic pathways eg in the generation of NFkB elements cRel and RelA which prevent TNFR-induced apoptosis (Liu et al., 1996) . Accordingly in the absence of CD28 ligation, ceramide might be utilized by Ras, driving apoptosis. Support for this hypothesis may be provided by testing the viability of C2 ceramide treated costimulated T cells in the presence of absence of wortmannin to assess whether the inhibition of PI3K activation prevents CD28 lysosomal translocation and therefore a functional association with ASMase, thereby possibly preventing a CD28-modulated use of ceramide. It might then be expected that ceramide would be available for use in a Ras-derived apoptotic pathway.

The action of ceramide on costimulated T cells has some similarity with that of CTLA4 in that both negatively regulate T cell activation and proliferation (Krummel and Allison,

1996). It may be possible that they share common effectors and/ or regulate the function of each other. These possibilities may be examined by using FACS analysis of CTLA4 expression following ceramide treatment of costimulated T cells. Additionally incubating CD3 stimulated T cells in exogenous ASMase +/ - chloroquine may indicate whether ASMase increases CTLA4 expression. It is also possible that CTLA4 utilizes ASMase in its signal transduction pathways and antibody ligation of CTLA4 in costimulated T cells in the presence or absence of chloroquine may reveal an involvement of ASMase in CTLA4 signalling. Another contender which may facilitate cross-talk between CD28 and CTLA4 is itk which has been associated with negative regulation of costimulated T cell proliferation (Liao et al., 1997). It is possible to speculate that itk may associate with ASMase/ ceramide in T cell signalling either to activate ASMase or to become activated by ASMase. In either case it would be interesting to perform in vitro kinase assays against immunoprecipitated itk from cell lysates and lysosomes to determine whether in costimulated T cell cultures ceramide had a stimulatory effect on itk activity. Secondly it may be interesting to determine whether itk associates with CTLA4 possibly giving CTLA4 the initial impetus to transduce negative regulation of T cell proliferation and activation. A possible role for itk in CTLA4 signalling may be to activate a PTPase resulting in the dephosphorylation of Y165 and so increasing the surface expression of CTLA4 (Shiratori et al., 1997). Alternatively itk might phosphorylate Y165, allowing the association of the PTPase Syp with CTLA4. As Syp has activity against SHC (Marengere et al., 1996), this would prevent SHC complexing with GRB2 and Sos thereby inhibiting Ras signalling.

There still remains a large area of CD28 signalling which has not yet been investigated and future studies may determine the relationship between CD28 and CTLA4, and putative relationships between ASMase/ ceramide, Ras and PI3K and CD28.

Chapter 8

Bibliography

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, JD. (1989). Chapter 13; Cell Growth and Division. Molecular Biology of the Cell *2nd Edition*,

Allison, J. (1994). CD28-B7 interactions in T cell activation. Current Opinion in Immunology *6*, 414-419.

Anel, A., Buferne, M., Boyer, C., Schmitt-Verhulst, A-M., and Golstein, P. (1994). T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosproin A. Eur. J. Immunol. *24*, 2469-2476.

Appleby, MW., Gross, JA., Cooke, MP., Levin, SD., Qian, X., and Perlmutter, RM. (1992). Defective T cell Receptor Signalling in Mice Lacking the Thymic Isoform of $p59^{fyn}$. Cell *70*, 751-763.

Aruffo, A. and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA *84*, 8573-8577.

Atkinson, EA., Ostergaard, H., Kane, K., Pinkoski, MJ., Caputo, A., Olszowy, MW., and Bleackley, C. (1996). A Physical Interaction between the Cell Death Protein Fas and the Tyrosine Kinase $p59^{fynT}$. J. Biol. Chem. *271*, 5968-5971.

August, A. and Dupont, B. (1994a). Activation of src family kinase lck following CD28 crosslinking in the Jurkat Leukemic cell line. Biochemical and Biophysical Research Communications *199*, 1466-1473.

August, A. and Dupont, B. (1994b). CD28 of T lymphocytes associates with phophatidylinositol 3-kinase. International Immunology *6*, 769-774.

August, A., Gibson, S., Kawakami, Y., Kawakami, T., Mills, GB., and Dupont, B. (1994). CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T cell line. Proc. Natl. Acad. Sci. USA *91*, 9347-9351.

Autero, M., Saharinen, J., Pessa-Morikawa, T., Soula-Rothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., Gahmberg, CG., and Mustelin, T. (1994). Tyrosine Phosphorylation of CD45 Phosphotyrosine Phosphatase by $p50^{csk}$ Kinase Creates a Binding Site for $p56^{lck}$ Tyrosine Kinase and Activates the Phosphatase. Mol. Cell. Biol *14*, 1308-1321.

Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, JH., Lanier, LL., and Somoza, C. (1993). B70 antigen is a second ligand for CTLA-4 and CD28. Nature *366*, 76-79.

Boise, LH., Minn, AJ., Noel, PJ., June, CH., Accavitti, MA., Lindsten, T., and Thompson, CB. (1995). CD28 Costimulation Can Promote T Cell Survival by Enhancing the Expression of Bcl-x_L. Immunity *3*, 87-98.

Boucher, L-M., Wiegmann, K., Futterer, A., Pfeffer, K., Machleidt, T., Schutze, S., Mak, TW., and Kronke, M. (1995). CD28 Signals Through Acidic Sphingomyelinase. J. Exp. Med. *181*, 2059-2068.

Boussiotis, V., Barber, DL., Lee, BJ., Gribben, JG., Freeman, GJ., and Nadler, LM. (1996). Differential Association of Protein Tyrosine Kinases with the T Cell Receptor Is Linked to the Induction of Anergy and Its Prevention by B7 Family-mediated Costimulation. J. Exp. Med. *184*, 365-376.

Bryan, RG., Li, Y., Lai, J-H., Van, M., Rice, NR., Rich, RR., and Tan, T-H. (1996). Effect of CD28 Signal Transduction on c-Rel in Human Peripheral Blood T Cells. Mol. Cell. Biol *14*, 7933-7942.

Burgering, BMT. and Coffer, PJ. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Cai, Y-C., Cefai, D., Schneider, H., Raab, M., Nabavi, N., and Rudd, CE. (1995). Selective CD28 pYMNM Mutations Implicate Phosphatidylinositol 3-Kinase in CD86-CD28-Mediated Costimulation. Immunity *3*, 417-426.

Camp, LA. and Hofmann, SL. (1993). Purification and Properties of a Palmitoyl-Protein Thioesterase That Cleaves Palmitate from H-Ras. J. Biol. Chem. *268*, 22566-22574.

Cefai, D., Schneider, H., Matangkasombut, O., Kang, H., Brody, J., and Rudd, CE. (1998). CD28 Receptor Endocytosis Is Targeted by Mutations That Disrupt Phosphatidylinositol 3-Kinase Binding and Costimulation. J. Immunol. *160*, 2223-2230.

Chan, AC., Kadlecek, TA., Elder, ME., Filipovich, AH., Kuo, W-L., Iwashima, M., Parslow, TG., and Weiss, A. (1994). ZAP-70 Deficiency in an Autosomal Recessive Form of Severe Combined Immunodeficiency. Science *264*, 1599-1601.

Chan, G. and Ochi, A. (1995). Sphingomyelin-ceramide turnover in CD28 costimulatory signaling. Eur. J. Immunol. *25*, 1999-2004.

Chatta, GS., Spies, AG., Chang, S., Mize, GJ., Linsley, PS., Ledbetter, JA., and Morris, DR. (1994). Differential Regulation of Proto-Oncogenes c-jun and c-fos in T Lymphocytes Activated Through CD28. J. Immunol. *153*, 5393-5401.

Cheng, G., Ye, Z-S., and Baltimore, D. (1994). Binding of Bruton's tyrosine kinase to Fyn, Lyn or Hck through a Src homology 3 domain-mediated interaction. Proc. Natl. Acad. Sci. USA *91*, 8152-8155.

Cifone, MG., De Maria, R., Roncaioli, P., Rippo, MR., Azuma, M., Lanier, LL., Santoni, A., and Testi, R. (1993). Apoptotic Signaling through CD95 (Fas/ Apo-1) Activates an Acidic Sphingomyelinase. J. Exp. Med. *177*, 1547-1552.

Clarke, WR. (1980). . The Experimental Foundations of Modern Immunology *Chapter 1, 2 and 7*,

Cooper, JA. and Whyte, P. (1989). RB and the Cell Cycle: Entrance or Exit? Cell *58*, 1009-1011.

Crooks, M.E.Casey, Littman, Dan R., Carter, Robert H., Fearon, Douglas T., Weiss, A., and Stein, P.H. (1995). CD28-Mediated Costimulation in the Absence of Phosphatidylinositol 3-Kinase Association and Activation. Mol. Cell. Biol *15*, 6820-6828.

Damle, NK., Klussman, K., Linsley, PS., and Aruffo, A. (1992). Differential Costimulatory Effects of Adhesion Molecules B7, ICAM-1, LFA-3 and VCAM-1 on Resting and Antigen-Primed CD4+ T lymphocytes. J. Immunol. *148*, 1985-1992.

Das, DVM., Cook, HW., and Spence, MW. (1984). Evidence That Neutral Sphingomyelinase of Cultured Murine Neuroblastoma Cells Is Orientated Externally on the Plasma Membrane. BBA *777*, 339-342.

Davis, MM. and Bjorkman, PJ. (1988). T-cell antigen receptor genes and T-cell recognition. Nature *334*, 395-402.

Dbaibo, GS., Pushkareva, MY., Jayadev, S., Schwarz, JK., Horowitxz, JM., Obeid, LM., and Hannun, YA. (1995). Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest. Proc. Natl. Acad. Sci. USA *92*, 1347-1351.

Desai, NN., Zhang, H., Olivera, A., Mattie, ME., and Spiegel, S. (1992). Sphingosine-1-phosphate, a Metabolite of Sphingosine, Increases Phosphatidic Acid Levels by Phospholipase D Activation. J. Biol. Chem. *267*, 23122-23128.

Dobrowsky, RT. and Hannun, YA. (1992). Ceramide Stimulates a Cytosolic Protein Phosphatase. J. Biol. Chem. *267*, 5048-5051.

Dobrowsky, RT., Kamibayashi, C., Mumby, M., and Hannun, YA. (1993). Ceramide Activates Heterotrimeric Protein Phosphatase 2A. J. Biol. Chem. *268*, 15523-15530.

Edmead, CE., Patel, YI., Wilson, A., Boulougouris, G., Hall, ND., Ward, SG., and Sansom, DM. (1996). Induction of Activator Protein (AP) -1 and Nuclear Factor-kB by CD28 Stimulation Involves Both Phosphatidylinositol 3-Kinase and Acidic Sphingomyelinase Signals. J. Immunol. *157*, 3290-3297.

Faris, M., Kokot, N., Lee, L., and Nel, AE. (1996). Regulation of Interleukin-2 Transcription by Inducible Stable Expression of Dominant Negative and Dominant Active Mitogen-activated Protein Kinase Kinase Kinase in Jurkat T Cells Evidence for the Importance of Ras in a Pathway that is Controlled by Dual Receptor Stimulation. J. Biol. Chem. *271*, 27366-27373.

Felding-Habermann, B., Igarashi, Y., Fenderson, BA., Park, LS., Radin, NS., Inokuchi, J-i., Strassmann, G., Handa, K., and Hakomori, S-i. (1990). A Ceramide Analogue Inhibits T Cell Proliferative Response through Inhibition of Glycosphingolipid Synthesis and Enhancement of N, N-Dimethylsphingosine Synthesis. Biochemistry *29*, 6314-6322.

Franke, TF., Yang, SI., Chan, TO., Datta, K., Kazlauskas, A., Morrison, DK., Kaplan, DR., and Tsichlis, PN. (1995). The Protein Kinase Encoded by the *Akt* Proto-Oncogene Is a Target of the PDGF-Activated Phosphatidylinositol 3-Kinase. Cell *81*, 727-736.

Fukami, K. and Takenawa, T. (1992). Phosphatidic Acid That Accumulates in Platelet-derived Growth Factor-stimulated Balb/c 3T3 Cells Is a Potential Mitogenic Signal. J. Biol. Chem. *267*, 10988-10993.

Germain, RN. and Marguiles, DH. (1993). The Biochemistry and Cell Biology of Antigen Processing and Presentation. Ann. Rev. Immunol. *Ch11*, 403-450.

Ghosh, TK., Bian, J., and Gill, DL. (1990). Intracellular Calcium Release Mediated by Sphingosine Derivatives Generated in Cells. Science *248*, 1653-1656.

Gibson, S., August, A., Branch, D., Dupont, B., and Mills, GB. (1996). Functional LCK is Required for Optimal CD28-mediated Activation of the TEC Family Tyrosine Kinase EMT/ ITK. J. Biol. Chem. *271*, 7079-7083.

Gibson, S., Truitt, K., Lapushin, R., Khan, H., Imboden, JB., and Mills, GB. (1998). Efficient CD28 signalling leads to increases in the kinase activities of the TEC family tyrosine kinase EMT/ITK/TSK and the SRC family tyrosine kinase LCK. Biochem. J. *330*, 1123-1128.

Gonzalez-Garcia, A., -Borlado, LR., Leonardo, E., Merida, I., Martinez-A., C., and Carrera, AC. (1997). Lck Is Necessary and Sufficient for Fas-Ligand Expression and Apoptotic Cell Death in Mature Cycling T Cells. J. Immunol. *158*, 4104-4112.

Granelli-Piperno, A. and Nolan, P. (1991). Nuclear Transcription Factors that Bind to Elements of the IL-2 Promoter. J. Immunol. *147*, 2734-2739.

Greene, JL., Leytze, GM., Emswiler, J., Peach, R., Bajorath, J., Cosand, W., and Linsley, PS. (1996). Covalent Dimerization of CD28/ CTLA-4 and Oligomerization of CD80/ CD86 Regulate T cell Costimulatory Interactions. J. Biol. Chem. *271*, 26762-26771.

Gulbins, E., Bissonnette, R., Mahboubi, A., Martin, S., Nishioka, W., Brunner, T., Baier, G., Baier-Bitterlich, G., Byrd, C., Lang, F., Kolesnick, R., Altman, A., and Green, D. (1995). FAS-Induced Apoptosis Is Mediated Via a Ceramide-Initaited RAS Signaling Pathway. Immunity *2*, 341-351.

Haimovitz-Friedman, A., Balaban, N., McLoughlin, M., Ehleiter, D., Michaeli, J., Vlodavsky, I., and Fuks, Z. (1994). Protein Kinase C Mediates Basic Fibroblast Growth Factor Protection of Endothelial Cells against Radiation-induced Apoptosis. Cancer Research *54*, 2591-2597.

Hakomori, S-i. (1990). Bifunctional Role of Glycosphingolipids. J. Biol. Chem. *265*, 18713-18716.

Hara, T., Fu, SM., and Hansen, JA. (1985). Human T cell Activation. J. Exp. Med. *161*, 1513-1524.

Harlan, DM., Abe, R., Lee, KP., and June, CH. (1995). Potential Roles of the B7 and CD28 Receptor Families in Autoimmunity and Immune Evasion. Clinical Immunology and Immunopathology *75*, 99-111.

Hatada, MH., Lu, X., Laird, ER., Green, J., Morgenstern, JP., Lou, M., Marr, CS., Phillips, TB., Ram, MK., Theriault, K., Zoller, MJ., and Karas, JL. (1995). Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. Nature *377*, 32-38.

Hazzalin, C., Cano, E., Cuenda, A., Barratt, MJ., Cohen, P., and Mahadevan, LC. (1996). p38/ RK is essential for stress-induced responses: JNK/ SAPKs and c-Jun/ ATF-2 phosphorylation are insufficient. Current Biology *6*, 1028-1031.

Higuchi, M., Singh, S., Jaffrezou, J-P., and Aggarwal, BB. (1996). Acidic Sphingomyelinase-Generated Ceramide is Needed But Not Sufficient for TNF-Induced Apoptosis and Nuclear Factor-kB Activation. J. Immunol. *156*, 297-304.

Hoffman-Ostenhoff, O., Cohn, WE., Braunstein, AE., Dixon, HBF., Horecker, BL., Jakoby, WB., Karlson, P., Klyne, W., Liebecq, C., and Webb, EC. (1978). The Nomenclature of Lipids. Biochem. J. *171*, 21-35.

Hutchcroft, JE. and Bierer, BE. (1994). Activation-dependent phosphorylation of the T-lymphocyte surface receptor CD28 and associated proteins. Proc. Natl. Acad. Sci. USA *91*, 3260-3264.

Hutchcroft, JE., Franklin, DP., Tsai, B., Harrison-Findik, D., Vartikovski, L., and Bierer, BE. (1995). Phorbol ester treatment inhibits phosphatidylinositol 3-kinase activation by, and association with, CD28, a T lymphocyte surface receptor. Proc. Natl. Acad. Sci. USA *92*, 8808-8812.

Hutchcroft, JE., Tsai, B., and Bierer, BE. (1996). Differential Phosphorylation of the T Lymphocyte Costimulatory Receptor CD28. J. Biol. Chem. *271*, 13362-13370.

Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S., van der Bosch, H., and Pfeilschifter, J. (1996). Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. Proc. Natl. Acad. Sci. USA *93*, 6959-6963.

Igarashi, Y., Hakomori, S-i., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989). Effect of Chemically Well-Defined Sphingosine and Its N-Methyl Derivates on Protein Kinase C and src Kinase Activities. Biochemistry *28*, 6796-6800.

Irving, BA. and Weiss, A. (1991). The Cytoplasmic Domain of the T Cell Receptor ζ Chain Is Sufficient to Couple to Receptor-Associated Signal Transduction Pathways. Cell *64*, 891-901.

Jarvis, WD., Fornari, FA., Browning, JL., Gewirtz, DA., Kolesnick, RN., and Grant, S. (1994). Attenuation of Ceramide-induced Apoptosis by Diglyceride in Human Myeloid Leukaemia Cells. J. Biol. Chem. *269*, 31685-31692.

Jayadev, S., Liu, B., Bielawska, AE., Lee, JY., Nazaire, F., Pushkareva, MY., Obeid, LM., and Hannun, YA. (1995). Role for Ceramide in Cell Cycle Arrest. J. Biol. Chem. *270*, 2047-2052.

Johns, L., Sarr, T., and Ranges, GE. (1994). Inhibition of Ceramide Pathway Does Not Affect Ability of TNF-a to Activate Nuclear Factor-kB. J. Immunol. *152*, 5877-5882.

June, CH., Bluestone, JA., Nadler, LM., and Thompson, CB. (1994). The B7 and CD28 receptor families. Immunol. Today *15*, 321-331.

Kaga, S., Ragg, S., Rogers, KA., and Ochi, A. (1998a). Activation of p21-CDC42/ Rac-Activated Kinases by CD28 Signaling: p21-Activated Kinase (PAK) and MEK Kinase 1 (MEKK1) May Mediate the Interplay Between CD3 and CD28 Signals. J. Immunol. *160*, 4182-4189.

Kaga, S., Ragg, S., Rogers, KA., and Ochi, A. (1998b). Stimulation of CD28 with B7-2 Promotes Focal Adhesion-Like Cell Contacts Where Rho FAmily Small G Proteins Accumulate in T Cells. Journal of Immunology *160*, 24-27.

Kanoh, H., Yamada, K., and Sakane, F. (1990). Diacylglycerol kinase: a key modulator of signal transduction? TIBS *15*, 47-50.

Kim, M-Y., Linardic, C., Obeid, L., and Hannun, Y. (1991). Identification of Sphingomyelin Turnover as an Effector Mechanism for the Action of Tumour Necrosis Factor a and g-Interferon. J. Biol. Chem. *266*, 484-489.

King, PD., Sadra, A., Teng, JMC., Liu, X-R., Han, A., Selvakumar, A., August, A., and Dupont, B. (1997). Analysis of CD28 Cytoplasmic Tail Tyrosine Residues as Regulators and Substrates for the Proetin Tyrosine Kinases, EMT and LCK. J. Immunol. *158*, 580-590.

Kolesnick, R. and Fuks, Z. (1995). Ceramide: A Signal for Apoptosis or Mitogenesis. J. Exp. Med. *181*, 1949-1952.

Krummel, MF. and Allison, JP. (1995). CD28 and CTLA-4 Have Opposing Effects on the Response of T cells to Stimulation. J. Exp. Med. *182*, 459-465.

Krummel, MF. and Allison, JP. (1996). CTLA-4 Engagement Inhibits IL-2 Accumulation and Cell Cycle Progression upon Activation of Resting T Cells. J. Exp. Med. *183*, 2533-2540.

Kuchroo, VK., Das, MP., Brown, JA., Ranger, AM., Zamvil, SS., Sobel, RA., Weiner, HL., Nabavi, N., and Glimcher, LH. (1995). B7-1 and B7-2 Costimulatory Molecules Activate Differentially the Th1/ Th2 Developmental Pathways: Application to Autoimmune Diesease Therapy. Cell *80*, 707-718.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, NM., and Hall, MN. (1993). Target of Rapamycin in Yeast, TOR2, is an Essential Phosphatidylinositol Kinase Homolog Required for G_1 Progression. Cell *73*, 585-596.

Kyriakis, JM., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, EA., Ahmad, MF., Avruch, J., and Woodgett, JR. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. Nature *369*, 156-160.

Lai, JH. and Tan, TH. (1994). CD28 Signalling Causes a Sustained Down-regulation of IkBa Which Can Be Prevented by the Immunosuppressant Rapamycin. J. Biol. Chem. *269*, 30077-30080.

Lakkis, FG., Konieczny, BT., Saleem, S., Baddoura, FK., Linsley, PS., Alexander, DZ., Lowry, RP., Pearson, TC., and Larsen, CP. (1997). Blocking the CD28-B7 T cell Costimulation Pathway Induces Long Term Cardiac Allograft Acceptance in the Absence of IL-4. Journal of Immunology *158*, 2443-2448.

Larsen, CP., Elwood, ET., Alexander, DZ., Ritchie, SC., Hendrix, R., Tucker-Burden, C., Cho, HR., Aruffo, A., Hollenbaugh, D., Linsley, PS., Winn, KJ., and Pearson, TC. (1996). Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature *381*, 434-438.

Leung, HT., Bradshaw, J., Cleaveland, JS., and Linsley, PS. (1995). Cytotoxic T Lymphocyte-associated Molecule-4, a High Avidity Receptor for CD80 and CD86, Contains an Intracellular Localization Motif in Its Cytoplasmic Tail. J. Biol. Chem. *270*, 25107-25114.

Liao, XC., Fournier, S., Killeen, N., Weiss, A., Allison, JP., and Littman, DR. (1997). Itk Negatively Regulates Induction of T Cell Proliferation by CD28 Costimulation. J. Exp. Med. *186*, 221-228.

Lin, R-H., Hwang, Y-W., Yang, B-C., and Lin, C-S. (1997). TNF Receptor-2 Triggered Apoptosis Is Associated with the Down-Regulation of Bcl-X_L on Activated T cells and Can Be Prevented by CD28 Costimulation. J. Immunol. *158*, 598-603.

Linsley, PS., Bradshaw, J., Greene, J., Peach, R., Bennett, KL., and Mittler, RS. (1996). Intracellular Trafficking of CTLA-4 and Focal Localization Towards Sites of TCR Engagement. Immunity *4*, 535-543.

Linsley, PS., Bradshaw, J., Urnes, M., Grosmaire, L., and Ledbetter, JA. (1993). CD28 Engagement by B7/BB-1 Induces Transient Down-Regulation of CD28 Synthesis and Prolonged Unreponsiveness to CD28 Signalling. J. Immunol. *150*, 3161-3169.

Linsley, PS., Brady, W., Urnes, M., Grosmaire, LS., Damle, NK., and Ledbetter, JA. (1991). CTLA-4 Is a Second Receptor for the B Cell Activation Antigen B7. J. Exp. Med. *174*, 561-569.

Liu, J. (1993). FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. Immunol. Today *14*, 290-295.

Liu, J., Mathias, S., Yang, Z., and Kolesnick, RN. (1994). Renaturation and Tumour Necrosis Factor-α Stimulation of a 97-kDa Ceramide-activated Protein Kinase. J. Biol. Chem. *269*, 3047-3052.

Liu, Z-g., Hsu, H., Goeddel, DV., and Karin, M. (1996). Dissection of the TNF Receptor 1 Effector Functions: JNK Activation Is Not Linked to Apoptosis While NFkB Activation Prevents Cell Death. Cell *87*, 565-576.

Lozano, J., Berra, E., Municio, MM., Diaz-Meco, MT., Dominguez, I., Sanz, L., and Moscat, J. (1994). Protein Kinase C ζ Isoform Is Critical for kB-dependent Promoter Activation by Sphingomyelinase. J. Biol. Chem. *269*, 19200-19202.

Lu, Y., Granelli-Piperno, A., Bjorndahl, JM., Phillips, CA., and Trevillyan, JM. (1992). CD28-Induced T Cell Activation. J. Immunol. *149*, 24-29.

Lu, Y., Phillips, CA., Bjorndahl, JM., and Trevillyan, JM. (1994). CD28 signal transduction: tyrosine phosphorylation and receptor association of phosphoinositide-3 kinase correlate with Ca^{2+}-independent costimulatory activity. Eur. J. Immunol. *24*, 2732-2739.

Lynch, DH., Ramsdell, F., and Alderson, MR. (1995). Fas and FasL in the homoeostatic regulation of immune responses. Immunol. Today *16*, 569-574.

Machleidt, T., Wiegmann, K., Henkel, T., Schutze, S., Baeuerle, P., and Kronke, M. (1994). Sphingomyelinase Activates Proteolytic IkB-a Degradation in a Cell-free System. J. Biol. Chem. *269*, 13760-13765.

Marengere, LEM., Okkenhaug, K., Clavreul, A., Couez, D., Gibson, S., Mills, GB., Mak, TW., and Rorrapel, R. (1997). The SH3 Domain of Itk/EMT Binds to Proline-Rich Sequences in the Cytoplasmic Domain of the T Cell Costimulatory Receptor CD28. Journal of Immunology *159*, 3220-3229.

Marengere, LEM., Waterhouse, P., Duncan, GS., Mittrucker, H-W., Feng, G-S., and Mak, TW. (1996). Regulation of T Cell Receptor Signaling by Tyrosine Phosphatase SYP Association with CTLA-4. Science *272*, 1170-1173.

Mathias, S., Dressler, K., and Kolesnick, R. (1991). Characterisation of a ceramide-activated protein kinase: Stimulation by tumour necrosis factor α. Proc. Natl. Acad. Sci. USA *88*, 10009-10013.

Mathias, S., Younes, A., Kan, C-C., Orlow, I., Joseph, C., and Kolesnick, RN. (1993). Activation of the Sphingomyelin Signaling Pathway in Intact EL4 Cells and in a Cell-Free System by IL-1b. Science *259*, 519-522.

McFarland, EDC., Hurley, TR., Pingel, JT., Sefton, BM., Shaw, A., and Thomas, ML. (1992). Correlation between Src family member regulation by the protein-tyrosine phosphatase CD45 and transmembrane signaling through the T-cell receptor. Proc. Natl. Acad. Sci. USA *90*, 1402-1406.

Merrill, AH., Nimkar, S., Menaldino, D., Hannun, Y., Loomis, C., Bell, RM., Tyagi, SR., Lambeth, JD., Stevens, VL., Hunter, R., and Liotta, DC. (1989). Structural Requirements for Long-Chain (Sphingoid) Base Inhibition of Protein Kinase C in Vitro and for the Cellular Effects of These Compounds. Biochemistry *28*, 3138-3145.

Minden, A., Lin, A., Claret, F-X., Abo, A., and Karin, M. (1995). Selective Activation of the JNK Signaling Cascade and c-Jun Transcriptional Activity by the Small GTPases Rac and Cdc42Hs. Cell *81*, 1147-1157.

Mueller, DL., Seiffert, S., Fang, W., and Behrens, TW. (1996). Differential Regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 Receptor in Cloned CD4+ Helper T Cells A Model for the Long-Term Survival of Memory Cells. J. Immunol. *156*, 1764-1771.

Mulligan, RC. and Berg, P. (1981). Selection for animal cells that express the *Escheria coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA *78*, 2072-2076.

Mustelin, T. (1994). T Cell Antigen Receptor Signalling: Three Families of Tyrosine Kinases and a Phosphatase. Immunity *1*, 351-356.

Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M., and Aizawa, S. (1993). Constitutive Activation of Src Family Kinases in Mouse Embryos That Lack Csk. Cell *73*, 1125-1135.

Nakanishi, H., Brewer, KA., and Exton, JH. (1993). Activation of the ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphophate. J. Biol. Chem. *268*, 13-16.

Ni, H-T., Deeths, MJ., and Mescher, MF. (1996). Phosphatidylinositol 3 Kinase Activity Is Not Essential for B7-1-Mediated Costimulation of Proliferation or Development of Cytotoxicity in Murine T Cells. J. Immunol. *157*, 2243-2246.

Nunes, J., Collette, Y., Truneh, H., Olive, D., and Cantrell, DA. (1994). The Role of $p21^{ras}$ in CD28 Signal Transduction: Triggering of CD28 with Antibodies but Not the Ligand B7-1, Activates $p21^{ras}$. J. Exp. Med. *180*, 1067-1076.

Nunes, J., Klasen, S., Franco, MD., Lipcey, C., Mawas, C., Bagnasco, M., and Olive, D. (1993). Signalling through CD28 T cell activation pathway involves an inositol phospholipid-specific phospholipase C activity. Biochem. J. *293*, 835-842.

Nunes, JA., Truneh, A., Olive, D., and Cantrell, DA. (1996). Signal Transduction by CD28 Costimulatory Receptor on T Cells. J. Biol. Chem. *271*, 1591-1598.

Olivera, A., Buckley, NE., and Spiegel, S. (1992). Sphingomyelinase and Cell-permeable Ceramide Analogs Stimulate Cellular Proliferation in Quiescent Swiss 3T3 Fibroblasts. J. Biol. Chem. *267*, 26121-26127.

Pages, F., Ragueneau, M., Klasen, S., Battifora, M., Couez, D., Sweet, R., Truneh, A., Ward, SG., and Olive, D. (1996). Two Distinct Intracytoplasmic Regions of the T Cell Adhesion Molecule CD28 Participate in Phosphatidylinositol 3-Kinase Association. J. Biol. Chem. *271*, 9403-9409.

Pages, F., Ragueneau, M., Rottapel, R., Truneh, A., Nunes, J., Imbert, J., and Olive, D. (1994). Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. Nature *369*, 327-329.

Pai, S-Y., Calvo, V., Wood, M., and Bierer, BE. (1994). Cross-linking CD28 leads to activation of 70-kDa S6 kinase. Eur. J. Immunol. *24*, 2364-2368.

Paige, LA., Nadler, MJS., Harrison, ML., Cassady, JM., and Geahlen, RL. (1993). Reversible Palmitoylation of the Protein Tyrosine Kinase p56^{lck}. J. Biol. Chem. *268*, 8669-8674.

Parra, E., Wingren, AG., Hedlund, G., Sjogren, H.-O., Kalland, T., Sansom, D., and Dohlsten, M. (1993). Human Naive and Memory T-Helper Cells Display Distinct Adhesion Properties to ICAM-1, LFA-3 and B7 Molecules. Scand. J. Immunol *38*, 508-514.

Parry, RV., Olive, D., Westwick, J., Sansom, DM., and Ward, SG. (1997). Evidence that a kinase distinct fron protein kinase C and phosphatidylinositol 3-kinase mediates ligation-dependent serine/ threonine phosphorylation of the T-lymphocyte co-stimulatory molecule CD28. Biochem. J. *326*, 249-257.

Prasad, KVS., Cai, YC., Raab, M., Duckworth, B., Cantley, L., Shoelson, SE., and Rudd, CE. (1994). T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. Proc. Natl. Acad. Sci. USA *91*, 2834-2838.

Price, DJ., Grove, JR., Calvo, V., Avruch, J., and Bierer, BE. (1992). Rapamycin-Induced Inhibition of the 70-Kilodalton S6 Protein Kinase. Science *257*, 973-976.

Pushkareva, M., Obeid, LM., and Hannun, YA. (1995). Ceramide: an endogenous regulator of apoptosis and growth supression. Immunol. Today *16*, 294-297.

Qiu, R-G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995). An essential role for Rac in Ras transformation. Nature *374*, 457-459.

Raab, M., Yun-Cai, C., Bunnell, SC., Heyeck, SD., Berg, LJ., and Rudd, CE. (1995). $p56^{lck}$ and $p59^{fyn}$ regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein GRB-2, and T cell-specific protein tyrosine kinase ITK: Implications for T cell costimulation. Proc. Natl. Acad. Sci. USA *92*, 8891-8895.

Raines, MA., Kolesnick, RN., and Golde, DW. (1993). Sphingomyelinase and Ceramide Activate Mitogen-activated Protein Kinase in Myeloid HL-60 Cells. J. Biol. Chem. *268*, 14572-14575.

Resh, Mr. (1994). Myristylation and Palmitylation of Src family Members: The Fats of the Matter. Cell *76*, 411-413.

Rincon, M. and Flavell, RA. (1994). AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. EMBO J. *13*, 4370-4381.

Robey, E. and Allison, JP. (1995). T cell activation: integration of signals from the antigen receptor and costimulatory molecules. Immunol. Today *16*, 306-310.

Rodriguez-Viciana, P., Warne, PH., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, MJ., Waterfield, MD., and Downward, J. (1994). Phosphatidlyinositol-3-OH kinase as a direct target of Ras. Nature *370*, 527-532.

Rudd, CE., Janssen, O., Cai, YC., Da Silva, AJ., Raab, M., and Prasad, KVS. (1994). Two-step TCRζ/CD3-CD4 and CD28 signaling in T cells: SH2/ SH3 domains, protein-tyrosine and lipid kinases. Immunol. Today *15*, 225-234.

Rulifson, IF., Sperling, AI., Fields, PE., Fitch, FW., and Bluestone, JA. (1997). CD28 Costimulation Promotes the Production of Th2 Cytokines. J. Immunol. *158*, 658-665.

Sabatini, DM., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, SH. (1994). RAFT1: A Mammalian Protein That Binds to FKBP12 in a Rapamycin-Dependent Fashion and is Homologous to Yeast TORs. Cell *78*, 35-43.

Sambrook, J., Fritsch, EF., and Maniatis, T. (1989). Molecular Cloning. A Laboratory Manual. Cold Spring Harbour Laboratory Press

Sanders, SE., Makgoba, MW., and Shaw, S. (1988). Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. Imm Tod *9*, 195-199.

Sansom, DM., Wilson, A., Boshell, M., Lewis, J., and Hall, ND. (1993). B7/CD28 but not LFA-3/CD2 interactions can provide 'third-party' co-stimulation for human T-cell activation. Immunology *80*, 242-247.

Santana, P., Pena, LA., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, EH., Fuks, Z., and Kolesnick, R. (1996). Acid Sphingomyelinase-Deficient Human Lymphoblasts and Mice Are Defective in Radiation Induced Apoptosis. Cell *86*, 189-199.

Schneider, H., Cai, YC., Prasad, KVS., Shoelson, SE., and Rudd, CE. (1995a). T cell antigen CD28 binds to the GRB-2/SOS complex, regulators of $p21^{ras}$. Eur. J. Immunol. *25*, 1044-1050.

Schneider, H., Prasad, KVS., Shoelson, SE., and Rudd, CE. (1995b). CTLA-4 Binding to the Lipid Kinase Phosphatidylinositol 3-Kinase in T Cells. J. Exp. Med. *181*, 351-355.

Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992). TNF Activates NF-kB by Phosphatidylcholine-Specific Phospholipase C-Induced "Acidic" Sphingomyelin Breakdown. Cell *71*, 765-776.

Schwartz, RH. (1993). T Cell Anergy. Scientific American *August*, 48-54.

Semnani, RT., Nutman, TB., Hochman, P., Shaw, S., and van Seventer, GA. (1994). Costimulation by Purified Intercellular Adhesion Molecule 1 and Lymphocyte Function-associated Antigen 3 Induces Distinct Proliferation , Cytokine and Cell Surface Antigen Profiles in Human "Naive" and "Memory" CD4+ T Cells. J. Exp. Med. *180*, 2125-2135.

Shahanian, A., Pfeffer, K., Lee, KP., Kundig, TM., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, PS., Thompson, CB., and Mak, TW. (1993). Differential T cell Costimulatory Requirements in CD28-Deficient Mice. Science *261*, 609-612.

Shenoy-Scaria, AM., Gauen, LKT., Kwong, J., Shaw, AS., and Lublin, DM. (1993). Palmitylation of an Amino-Terminal Cysteine Motif of Protein Tyrosine Kinases $p56^{lck}$ and $p59^{fyn}$ Mediates Interaction with Glycosyl-Phosphatidylinositol-Anchored Proteins. Mol. Cell. Biol *13*, 6385-6392.

Shiratori, T., Miyatake, S., Ohno, H., Nakaseko, C., Isono, K., Bonifacino, JS., and Saito, T. (1997). Tyrosine Phosphorylation Controls Internalization of CTLA-4 by

Regulating Its Interaction with Clathrin-Associated Adaptor Complex AP-2. Immunity *6*, 583-589.

Smazel, M. and Resch, K. (1995). T-cell antigen receptor-induced signal-transduction pathways Activation and function of protein kinases C in T lymphocytes. Eur. J. Biochem. *228*, 1-15.

Smith, KA. (1984). Interleukin 2. Ann. Rev. Immunol. *2*, 319-333.

Songyang, Z., Shoelson, SE., Chaudhuri, M., Gish, G., Pawson, T., Haser, WG., King, F., Roberts, T., Ratnofsky, S., Lechleider, RJ., Neel, BG., Birge, RB., Fajardo, JE., Chou, MM., Hanafusa, H., Schafhausen, B., and Cantley, LC. (1993). SH2 Domains Recognize Specific Phosphopeptide Sequences. Cell *72*, 767-778.

Spence, MW. (1993). Sphingomyelinases. Advances in Lipid Research *26*, 3-23.

Stein, PH., Fraser, JD., and Weiss, A. (1994). The Cytoplasmic Domain of CD28 Is both Necessary and Sufficient for Costimulation of Interleukin-2 Secretion and Association with Phosphatidylinositol 3'-Kinase. Mol. Cell. Biol *14*, 3392-3402.

Straus, DB. and Weiss, A. (1993). The CD3 Chains of the T cell Antigen Receptor Associate with the ZAP-70 Tyrosine Kinase and Are Tyrosine Phosphorylated after Receptor Stimulation. J. Exp. Med. *178*, 1523-1530.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994). JNK Is Involved in Signal Integration during Costimulation of T Lymphocytes. Cell *77*, 727-736.

Thompson, CB., Lindsten, T., Ledbetter, JA., Kunkel, SL., Young, HA., Emerson, SG., Leiden, JM., and June, CH. (1989). CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/ cytokines. Proc. Natl. Acad. Sci. USA *86*, 1333-1337.

Tivol, EA., Borriello, F., Schweitzer, AN., Lynch, WP., Bluestone, JA., and Sharpe, AH. (1995). Loss of CTLA-4 Leads to Massive Lymphoproliferation and Fatal Multiorgan Tissue Destruction, Revealing a Critical Negative Regulatory Role of CTLA-4. Immunity *3*, 541-547.

Truitt, KE., Shi, J., Gibson, S., Segal, LG., Mills, GB., and Imboden, JB. (1995). CD28 Delivers Costimulatory Signals Independently of Its Association with Phosphatidylinositol 3-Kinase. Immune Cell *155*, 4702-4710.

Tsai, M-H., Yu, C-L., Wei, F-S., and Stacey, DW. (1989). The Effect of GTPase Activating Protein upon Ras Is Inhibited by Mitogenically Responsive Lipids. Science *243*, 522-526.

Ueda, Y., Levine, BL., Huang, ML., Freeman, GJ., Nadler, LM., June, CH., and Ward, SG. (1995). Both CD28 ligands CD80 (B7-1) and CD86 (B7-2) activate phosphatidylinositol 3-kinase, and wortmannin reveals heterogeneity in the regulation of T cell IL2 secretion. International Immunology *7*, 957-966.

Uehara, Y., Fukazawa, H., Murakami, Y., and Mizuno, S. (1989). Irreversible Inhibition of V-src Tyrosine Kinase Activity by Herbimycin A and Its Abrogation by Sulfhydryl Compounds. Biochemical and Biophysical Research Communications *163*, 803-809.

Umlauf, SW., Beverly, B., Kang, SM., Brorson, K., Tran, AC., and Schwartz, RH. (1993). Molecular regulation of the IL-2 Gene: Rheostatic Control of the Immune System. Immunological Reviews *133*, 177-197.

Van Parijs, L., Ibraghimov, A., and Abbas, AK. (1996). The Roles of Costimulation and Fas in T Cell Apoptosis and Peripheral Tolerance. Immunity *4*, 321-328.

Vance, DE. and Vance, J. (1991). Biochemistry of Lipids, Lipoproteins and Membranes. Elsevier Science Publishers

Vandenberghe, P., Freeman, GJ., Nadler, LM., Fletcher, MC., Kamoun, M., Turka, LA., Ledbetter, JA., Thompson, CB., and June, CH. (1992). Antibody and B7/BB1-mediated Ligation of the CD28 Receptor Induces Tyrosine Phosphorylation in Human T cells. J. Exp. Med. *175*, 951-960.

Verheij, M., Bose, R., Lin, XH., Yao, B., Jarvis, WD., Grant, S., Birrer, MJ., Szabo, E., Zon, LI., Kyriakis, JM., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, RN. (1996). Requirement for ceramide-initiated SAPK/ JNK signalling in stress induced apoptosis. Nature *380*, 75-79.

Wadstrom, T. and Mollby, R. (1971). Studies on Extracellular Proteins from Staphylococcus aureus VI. Production and Purification of b-Haemolysin in Large Scale. BBA *242*, 288-307.

Walunas, TL., Bakker, CY., and Bluestone, JA. (1996). CTLA-4 Ligation Blocks CD28-dependent T Cell Activation. J. Exp. Med. *183*, 2541-2550.

Walunas, TL., Lenschow, DJ., Bakker, CY., Linsley, PS., Freeman, GJ., Green, JM., Thompson, CB., and Bluestone, JA. (1994). CTLA-4 Can Function as a Negative Regulator of T Cell Activation. Immunity *1*, 405-413.

Ward, SG., Reif, K., Ley, S., Fry, MJ., Waterfield, MD., and Cantrell, DA. (1992). Regulation of Phosphoinositide Kinases in T Cells. J. Biol. Chem. *267*, 23862-23869.

Ward, SG., Westwick, J., Hall, ND., and Sansom, DS. (1993). Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/ CD3 activation. Eur. J. Immunol. *23*, 2572-2577.

Ward, SG., Wilson, A., Turner, L., Westwick, J., and Sansom, DM. (1995). Inhibition of CD28-mediated T cell costimulation by the phosphoinositide 3-kinase inhibitor wortmannin. Eur. J. Immunol. *25*, 526-532.

Wiegmann, K., Schutze, S., Machleidt, T., Witte, D., and Kronke, M. (1994). Functional Dichotomy of Neutral and Acidic Sphingomyelinases in Tumour Necrosis Factor Signaling. Cell *78*, 1005-1015.

Williams, N. (1996). T Cell Inactivation Linked to Ras Block. Science *271*, 1234.

Wilson, E., Wang, E., Mullins, RE., Uhlinger, DJ., Liotta, DC., Lambeth, DJ., and Merill, AH. (1988). Modulation of the Free Sphingosine Levels in Human Neutrophils by Phorbol Esters and Other Factors. J. Biol. Chem. *263*, 9304-9309.

Wolff, RA., Dobrowsky, RT., Bielawska, A., Obeid, LM., and Hannun, YA. (1994). Role of Ceramide-activated Protein Phosphatase in Ceramide-mediated Signal Transduction. J. Biol. Chem. *269*, 19605-19609.

Yang, Z., Costanzo, M., Golde, DW., and Kolesnick, RN. (1993). Tumour Necrosis Factor Activation of the Sphingomyelin Pathway Signals Nuclear Factor kB Translocation in Intact HL-60 Cells. J. Biol. Chem. *268*, 20520-20523.

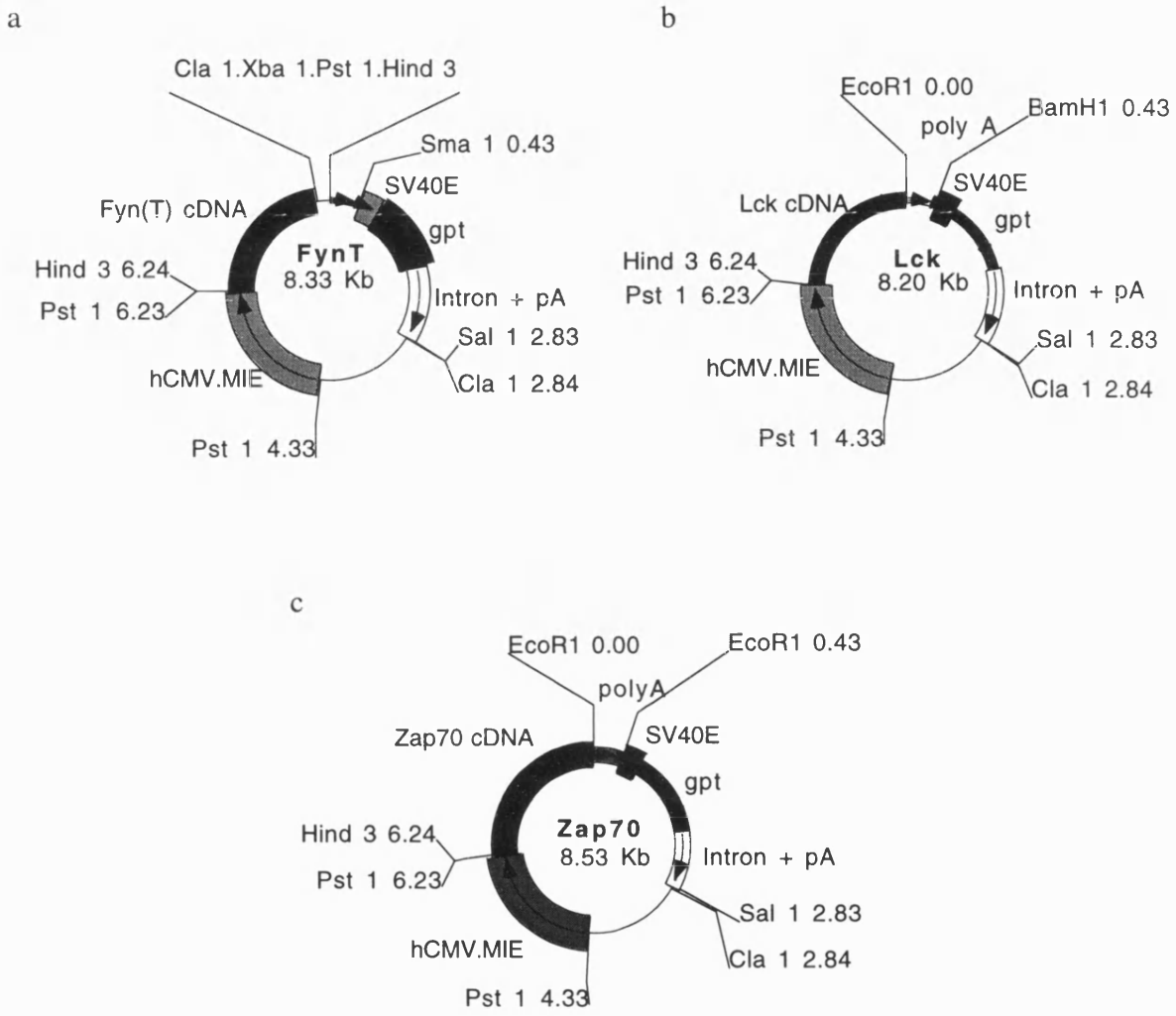
Yao, R. and Cooper, GM. (1995). Requirement for Phosphatidylinositol-3 Kinase in the Prevention of Apoptosis by Nerve Growth Factor. Science *267*, 2003-2005.

Zhang, H., Buckley, NE., Gibson, K., and Spiegel, S. (1990). Sphingosine Stimulates Cellular Proliferation via a Protein Kinase C-independent Pathway. J. Biol. Chem. *265*, 76-81.

Chapter 9

Appendices

Appendix 1- Plasmids



Representation of Plasmids Used to Transfect CD28+CHO Cells

a) fyn expression vector; b) lck expression vector c) ZAP expression vector.

Appendix 2 Buffers

1) HBS for Transfection

20mM HEPES	The solution was brought to pH 7.05 with HCl
137mM NaCl	and filtered sterilized
5mM KCl	
0.7mM Na_2HPO_4	
6.0mM D-Glucose	

2)Tris Buffered Saline (TBS)

20mM Tris.HCl pH 7.5	2.42g
150mM NaCl	17.52g
H_2O	to 1L

3)Phosphate Buffered Saline(PBS)

NaCl	8g
KCl	0.2g
NaH_2PO_4	0.2g
Na_2HPO_4	1.15g
H_2O	to 1L

4) CD28 IVK Reaction Buffer

100mM NaCl	1ml of 5M stock
25mM Hepes pH7.5	1.25mls of 1M stock
5mM $MnCl_2$	250µl of 1M stock
10mM $MgCl_2$	500µl of 1M stock
100µM Na_3VO_4	10µl of 0.5M stock
10µM MgATP	5µl of 100mM stock
H_2O	to 50mls

5) JNK Reaction Buffer

25mM Hepes pH 7.4	1.25mls of 1M stock
10mM MgAc	107mg
50µM MgATP	25µl of 100mM stock
H_2O	to 50mls

6) Coomassie Stain

0.25%w/v Coomassie Brilliant Blue R	1.25g (omitted for destaining solution)
45% v/v Methanol	225mls
10% v/v Glacial Acetic Acid	50mls
H_2O	to 500mls

Appendix 3 Cell Culture Media

1) R10 for Resting T cells

1 x RPMI 1640	500 mls (Life Technologies Cat. No 31870-025)
10 % FCS	50 mls (Life Tech. Cat. No 10108-074)
100 U/ ml Penicillin	5 mls of 10000 U/ ml
0.1 mg/ ml Streptomycin	5 mls of 10 mg/ ml stock(Life Tech. Cat. No 15140-114)
2 mM Glutamine	5 mls of 200 mM stock (Life Tech. Cat. No 25030-024)

2) R10 for Jurkat cells

1 x RPMI 1640	50 mls of 10x stock (Life Tech. Cat. No 22511-026)
10 % FCS	50 mls
100 U/ ml Penicillin	5 mls of 10000 U/ ml
0.1 mg/ ml Streptomycin	5 mls of 10 mg/ ml stock
2 mM Glutamine	5 mls of 200 mM stock
0.2 % Sodium bicarbonate	15 mls of 7.5 % stock (Life Tech. Cat No 25080-060)
0.015 N Sodium hydroxide	0.8 mls of 10 N stock
MilliQ H_2O	400 mls

3) Glutamine Free Media for CHO Transfectants

1x DMEM	50 mls of 10x stock (Life Tech. Cat. No 12501-029)
10% FCS	50 mls
100 U/ml Penicillin	5 mls of 10000 U/ ml
0.1 mg/ ml Streptomycin	5 mls of 10 mg/ ml
0.4 % Sodium bicarbonate	28 mls of 7.5 % stock
1 mM Sodium pyruvate	5 mls of 100 mM stock (Life Tech. Cat. No 11360-039)
1x Nucleosides	5 mls 0f 100x stock (see below)
MilliQ H_2O	400 mls

Appendix 3 Cell Culture Media continued

4) XMAT for PTK Transfected CHO Cells

1x DMEM	50 mls of 10x stock (Life Tech. Cat. No 12501-029)
10% FCS	50 mls
100 U/ml Penicillin	5 mls of 10000 U/ ml
0.1 mg/ ml Streptomycin	5 mls of 10mg/ ml
0.4 % Sodium bicarbonate	28 mls of 7.5% stock
1 mM Sodium pyruvate	5mls of 100mM stock (Life Tech. Cat. No 11360-039)
1x Nucleosides	5mls 0f 100x stock (see below)
1x XMAT	(see below)
MilliQ H_2O	400 mls

5) Nucleosides

These were dissolved in a combined volume of 100mls Milli-Q H_20 and filter sterilized.

	For CHO and CD28+CHOs in Glu- media	For PTK+Transfectants in XMAT media
Uridine	-	70mg
Guanosine	70mg	-
Adenosine	70mg	70mg
Cytosine	70mg	70mg
Thymidine	24mg	24mg

6) XMAT Components

50x Xanthine	1250 mg in 20 mls 1M NaOH + 80 mls H_20
100x Mycophenolic acid	150 mg in 10 mls 1M NaOH + 90 mls H_20
100x Hypoxanthine	150 mg in 10 mls 1M NaOH + 90 mls H_20
100x Thymidine	100mg in 100 mls H_20

All components were stored separately and 5mls of mycophenolic acid, hypoxanthine and thymidine and 10 mls of xanthine were added to XMAT media.

Appendix 4 Buffer Solutions

1) 10x Tris Acetate EDTA (TAE) pH 8.0

0.4M Tris base	48.44g
0.05M NaAc	4.1g
0.01M EDTA	3.72g
H_2O	to 1L

2) 1x SDS Gel Running Buffer pH8.3

25mM Tris base	3.03g
192mM Glycine	14.42g
0.1% w/v SDS	1g
H_2O	to 1L

3) 1x Lysis Buffer

50mM Tris.HCl, pH 7.5	0.5 mls of 1M
150mM NaCl	0.3 mls of 5M
1% v/v Nonidet P-40	1 ml of 10% v/v NP-40
10% v/v Glycerol	1 ml of 100%
5mM EDTA	100 µl of 0.5M pH8
1mM Na orthovanadate	100 µl of 0.1M
1mM Na molybdate	100 µl of 0.1M
10mM Na fluoride	200 µl of 0.5M
80µg/ml PMSF	10 µl of 80mg/ml (in ethanol)
1.4µg/ml Pepstatin A	10 µl of 1.4mg/ml (in methanol)
20µg/ml Aprotinin	10 µl of 20mg/ml
20µg/ml Leupeptin	10 µl of 20mg/ml
20µg/ml Soybean trypsin inhibitor	10 µl of 20mg/ml
H_20	to 10mls

4) 2x Loading Buffer

Glycerol	1 ml
10% w/v SDS	3 mls
0.5M Tris.HCl, pH 6.8	5 mls
0.5% w/v Bromophenol Blue	1 ml
β- mercaptoethanol	0.5 mls

Appendix 5 Gels

1) 1% Agarose Gel

1% agarose	1.5g
1x TAE	150mls
0.005% w/v Ethidium bromide	7.5 µl

2) 4% SDS-PAGE Stacking Gel

0.125M Tris.HCl	2.5mls of 0.5M stock pH 6.6
4% Acrylamide	1.33mls of 30% 37.5: 1 acrylamide: bis stock
0.1% w/v SDS	0.1mls of 10% w/v stock
0.15% w/v Ammonium persulphate	0.15mls of 10% w/v stock
0.003% v/v TEMED	30µl
H_2O	to 10mls

3) 12% SDS-PAGE Resolving Gel

0.375M Tris.HCl	5mls of 1.5M stock pH 8.8
12% Acylamide	8mls of 30% 37.5: 1 acrylamide: bis stock
0.1% w/v SDS	0.2mls of 10% w/v stock
0.05% w/v Ammonium persulphate	0.1mls of 10% w/v stock
0.0005% v/v TEMED	10µl
H_2O	to 20mls

Note: For SDS-PAGE, 1mm thick gels were prepared with a stacking gel of 1cm depth and samples were separated at a constant current of 10mA/ gel and through the resolving gel at 20mA/ gel.

Appendix 6 Bacterial Culture

1) Liquid Broth

Tryptone	10g
Yeast extract	5g
NaCl	10g
H_2O	to 1L

2) Agar Plates

Liquid broth	100mls
Agar	2g

Ampicillin may be added at 50μg/ ml following autoclaving if required once the mixture had cooled to 50°C before the plates are poured. 20mls/ plate was allowed.

3) SOC media

Tryptone	20g
Yeast extract	5g
NaCl	0.5g
Glucose	20mM
H_2O	to 1L